NEURAL RESPONSES TO INJURY: PREVENTION, PROTECTION, AND REPAIR
Annual Technical Report
1994

Submitted by

Nicolas G. Bazan, M.D., Ph.D.
Project Director

Period Covered: 20 September, 1993, through 19 September, 1994

Cooperative Agreement DAMD17-93-V-3013

between

United States Army Research and Development Command
(Walter Reed Army Institute of Research)

and

Louisiana State University Medical Center
Neuroscience Center of Excellence

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Neuropharmacology of Delta Receptor Agonists and Antagonists

Project Director
Joseph Moerschbaecher, Ph.D.

Participating Scientists
Charles France, Ph.D.
Dennis J. Paul, Ph.D.
Jayaraman Rao, M.D.

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6. AUTHOR (S)
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Professor of Ophthalmology, Biochemistry and Molecular Biology and Neurology

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13. ABSTRACT *(Maximum 200 words)*

The LSU Neuroscience Center is a comprehensive, multidisciplinary, and transdepartmental entity that unites fundamental neurobiology and the clinical neurosciences in the common goal of elucidating the workings of the brain and contributing to the treatment of currently incurable diseases of the nervous system. The objective of the present program is to find solutions to neuroscience-related problems of interest to the U.S. Army Medical Research and Development Command. The program is focused on exploiting novel neuroprotective strategies that lead to prevention of and repair after neural injury. Converging approaches using state-of-the-art tools of cell biology, neurochemistry, neuroimmunology, neurophysiology, neuropharmacology, molecular biology and virology are proposed. Over the next four years, this program aims to: 1) carry out seven research projects in the basic and clinical neurosciences; 2) expand central, shared facilities with the addition of highly specialized instrumentation not currently available to our scientists; 3) develop laboratory space to permit the physical consolidation and coordination of this research effort; and 4) institute a coordination unit to monitor, facilitate, and administrate the cooperative research programs, as well as to meet the associated budgetary, human resources, facilities, and communications needs for the attainment of the proposed program goals.

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This Technical Report covers the progress made in the first year of this Cooperative Agreement in one project of the original proposal. We hope that this format of the report will facilitate its handling. The table of contents for all the projects has been included in each volume as well as letters from members of the External Advisory Committee of the LSU Neuroscience Center who have conducted an initial review of the work done supported by this Cooperative Agreement.

Nicolas G. Bazan, M.D., Ph.D.
Director, LSU Neuroscience Center
Program Director, USAMRDC Cooperative Agreement

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Project Directors: Bryan Gebhardt, Ph.D.
Daniel Carr, Ph.D.

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2. Mouse lymphocytes express an orphan opioid receptor
3. Morphine suppresses peritoneal and splenic CTL activity in a dose-dependent fasion in alloimmunized mice
4. The frequency of exposure to morphine differentially affects CTL activity in alloimmunized mice.

Manuscripts:

1. Carr DJJ, Carpenter GW, Garza HH, Baker ML, Gebhart BM (in press) Cellular mechanisms involved in morphine-mediated suppression of CTL activity. In: *The Brain Immune Axis in Substance Abuse* (Sharp, Friedman, Maddin and Eisenstein, eds), Plenum Press.
2. Carpenter GW and Car DJJ (submitted) Pretreatment with β-funaltrexamine blocks morphine-mediated suppression of CTL activity in alloimmunized mice.
3. Carr DJJ and Carpenter GW (submitted) Morphine-induced suppression of spenic CTL activity in alloimmunized mice is not mediated through a δ-opioid receptor.
4. Carpenter GW, Garza HH, Gebhardt BM, Car DJJ (in press) Chronic morphine treatment suppresses CTL-mediated cytolysis, granulation and cAMP responses to alloantigen.

"Neurochemical Protection of the Brain, Neural Plasticity and Repair" . . . . . .

Project Director: Nicolas G. Bazan, M.D., Ph.D.

Participating Scientists: Geoffrey Allen, Ph.D.
Gary D. Clark, M.D.
Victor Marcheselli, M.S.
John Hurst, Ph.D.
Leo Happel, M.D.
Walter Lukiw, Ph.D.

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1. International Symposium on Nicotine: The Effects of Nicotine on Biological Systems II: Bienvenu B, Kiba H, Rao J, and Jayaraman A. Nicotine induceds fos intensely in the parvocellular paraventricular nucleus and the lateral hypothalamus in rats.

Figures 1 and 2

"Vision, Laser Eye Injury, and Infectious Diseases" . . . . .

Project Director: Herbert E. Kaufman, M.D.
Roger Beuerman, Ph.D.

Participating Scientists: Claude A. Burgoyne, M.D.
Emily Varnell
Mandi Conway, M.D.

Manuscripts

1. Chew SJ, Beuerman RW, Kaufman HE (in press) Real-time confocal microscopy of keratocyte activity in wound-healing after cryoablation in rabbit corneas. *Scanning* 16.

"Role of Growth Factors and Cell Signaling in the Response of Brain and Retina to Injury" . . . . .

Project Directors: Prescott Deininger, Ph.D.
Nicolas G. Bazan, M.D., Ph.D.

Participating Scientists: Julia Cook, Ph.D.
Haydee E. P. Bazan, Ph.D.

William C. Gordon, Ph.D.
Elena Rodriguez De Turco, Ph.D.
Victor Marcheselli, M.S.

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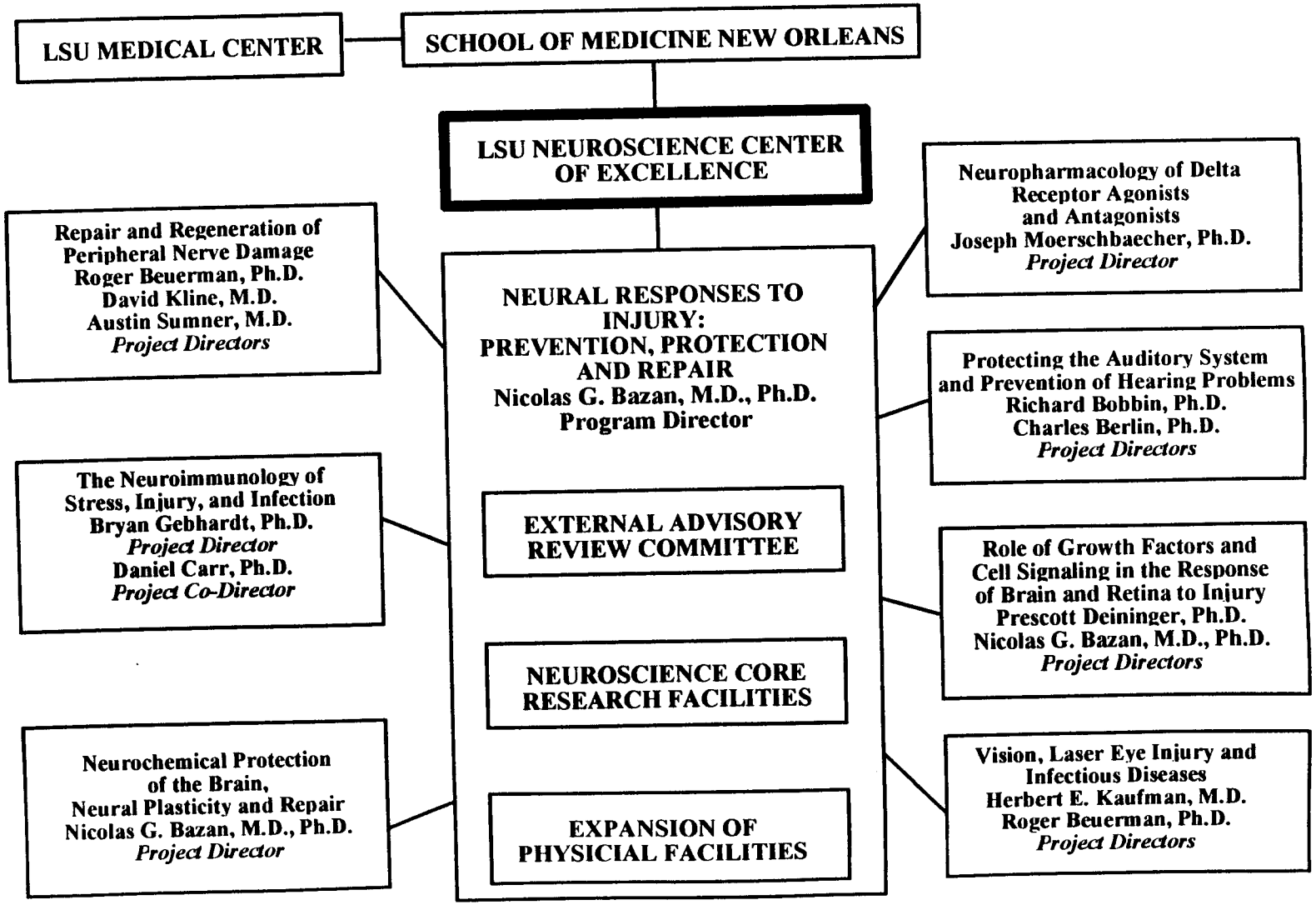
Cooperative Agreement Between the US Army Medical Research and Development Command and The LSU Neuroscience Center of Excellence

DAMD17-93-V-3013 20 September, 1993 - 19 October, 1997 $13,860,000



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Telefax: (504) 568-5801

Neuroscience Center
Office of the Director

19 October, 1994

Commander
U.S. Army Medical Research and Development Command (USAMRDC)
ATTN: SGRD-RMI-S
Fort Detrick
Frederick, MD 21702-5012

Re: Annual report, Cooperative Agreement No. DAMD17-93-V-3013
Neural Responses to Injury: Prevention, Protection, and Repair

Dear Sir,

Please find enclosed the original and five copies of the first annual report for the Cooperative Agreement, referenced above, between the USAMRDC and the Louisiana State University Medical Center School of Medicine, Neuroscience Center of Excellence. This report represents the research carried out during the first year of this agreement (20 September, 1993, to date). It is organized per project, each corresponding to a chapter of the original application.

In addition to the research conducted in the first year of this agreement, the planning for the two additional floors of research space which are to be added to the Lions/LSU Clinics Building, 2020 Gravier Street, New Orleans, LA, has been completed, including all specifications necessary for the start of bidding. Enclosed is one copy each of the program manual (1 vol.) and the project manual (3 vols.) which has been generated by Cimini, Meric and Duplantier, Architects and Planners, for bidding purposes. It should be noted that there will actually be three floors constructed in this one project, two as funded by this Cooperative Agreement and one which is funded by LSU to be used by the School of Medicine for other purposes.

As planned, I arranged to have three meetings between the LSU investigators and their counterparts in the Army to provide program briefings for the work that they were planning to conduct under this agreement as well as to exchange ideas and information of mutual interest. The agendas for each of these meetings are enclosed. These provided both the LSU scientists and those of the Army the opportunity to discuss the work being done, the direction, and the significance to problems of interest to the Department of Defense.

On 2 December, 1993, several of our investigators, excluding the Auditory and Laser/Vision groups, met at the Walter Reed Army Institute of Research, Washington, D.C., with Drs. Frank Tortella, Joseph Long, Mark DeCoster and Jit Dave. These discussions revolved around the neurochemical and neuropharmacological aspects of the program project and provided a forum for the Army scientists tobegin interactions and exchange of information with our investigators.

On 31 January, 1994, the LSU auditory physiology group, represented by Drs. Charles Berlin and Richard Bobbin, and I met at Fort Rucker, AL, with Dr. Kent Kimball and Dr. Ben T. Mozo. These meetings involved presentations and discussions about the protection of the auditory system and prevention of hearing problems in humans.

The LSU investigators involved with the vision research, composed of Dr. Herbert Kaufman, Dr. Roger Beuerman and myself, met on 7 February, 1994, at Brooks Air Force Base, San Antonio, TX. These scientists and those of the Ocular Hazards Research Unit of the US Army Medical Research Detachment made presentations and conducted discussions focused on protection from, repair of, and prevention of laser injuries, specifically to the eye. Each of these information exchanges provided very useful direction and advice for the LSU investigators. These workshops will be conducted annually for the term of this agreement.

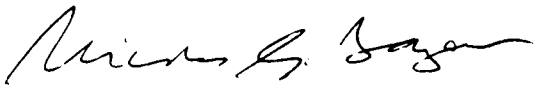
At the end of the first year of this program, as planned, I requested that two of the members of the External Advisory Committee of the LSU Neuroscience Center, Dr. Dennis W. Choi, Jones Professor and Head of the Department of Neurology, Washington University School of Medicine, and Dr. Fred Plum, Anne Parrish Titzell Professor and Chairman of the Department of Neurology, Cornell University Medical College, provide a critical review and a written report of the progress of the research accomplished under this Cooperative Agreement. Dr. Choi was given a copy of this annual report and subsequently made a site visit on 15 September, 1994, to the LSU Neuroscience Center. (The agenda for his meeting is attached.) At that time he met with a number of the investigators and administrators involved with whom he discussed many facets of the research being performed under this Agreement. His opinion of the work being done is attached.

Dr. Fred Plum made a site visit on 26 September, 1994, having also been provided previously with a copy of this annual report. He was also given the opportunity to examine the research and other progress made under this agreement and his written critique is also attached. Please note that, near the end of his letter (bottom of page two, first four paragraphs of page 5), Dr. Plum also included a description of projects not directly supported by the Cooperative Agreement but which are very positively impacted by any support of Neuroscience projects. The

reviewers were very complimentary of the positive consequences resulting from this support.

We are very pleased with the progress that has been made. We would like to thank you for the assistance you have given us. Please let me know if there is any further information that I can provide you.

Sincerely,



Nicolas G. Bazan, M.D., Ph.D.
Villere Professor of Ophthalmology,
Biochemistry and Molecular Biology,
and Neurology
Director, LSU Neuroscience Center

NGB/eht
enclosures

JOINT WORKSHOP ON "NEURAL RESPONSES TO INJURY: PREVENTION, PROTECTION AND REPAIR"

Sponsored by the LSU Neuroscience Center and Walter Reed Army Institute of Research, Department of Medical Neurosciences

December 2, 1993
Building 40, Room 2133

"Overview of LSU Program" **N. Bazan**	9:00
"Repair and Regeneration of Peripheral Nerve Damage" **R. Beuerman, D. Kline, J. England**	9:20
"The Neuroimmunology of Stress, Injury and Infection" **D. Carr**	10:10
Break	10:20
"Neurochemical Protection of the Brain, Neural Plasticity and Repair" **N. Bazan**	10:40
"Neuropharmacology of Delta Receptor Agonists and Antagonists" **J. Moerschbaecher**	11:15
"Stress and the Dopamine System" **J. Rao**	11:45
Box Lunch Served ($2.00 each)	12:00
"Role of Growth Factors and Cell Signaling in the Response of Brain and Retina to Injury **N. Bazan and J. Cook**	12:10
"An Overview of Neuropharmacology Research at WRAIR on Nervous System Injury and Protection" **Frank Tortella**	13:00
"Animal Models of Spinal Cord Injury and Mechanisms of Blood Flow Changes" **Joseph Long**	13:30
"Evaluation of Excitatory Amino Acids in Neuronhal Cell Culture" **CPT DeCoster**	13:50
"Molecular Biology of Nervous System" **Jit Dave**	14:10
Overall Discussion	14:30
Adjourn	15:00

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Joint Workshop on Neural Responses to Injury:
Prevention, Protection and Repair
Walter Reed Army Institute of Research, Dept. of Medical Neuroscience
U.S. Army Aeromedical Research Laboratory, Fort Rucker, AL
SCHEDULE FOR JANUARY 31, 1994

January 30

12:00 PM - depart New Orleans by car

Hotel: **Comfort Inn, 615 Boll Weevil Circle, Enterprise, AL 36330**
Tel. 205-393-2304, Fax. 205-347-5954

January 31

Visiting - Dr. Kent Kimball, Director, Plans and Programs, USAARL
Dr. Ben T. Mozo, Research Physicist, USAARL
Fort Rucker, AL 36362-5292
Tel. (205) 255-6917, Fax. (205) 255-6937

9:00 AM - Welcome
9:20 AM - Overview of LSU Program - **Nicolas G. Bazan**
9:45 AM - Protection the Auditory System and Prevention of Hearing Problem via Efferent Activation in Humans - **Charles Berlin**
10:30 AM - Break
11:00 AM - Prevention of Hearing Problems in Animals - **Richard Bobbin**
12:00 PM - General Discussion and Lunch
13:00 PM - Adjourn

OCULAR HAZARDS RESEARCH
U.S. ARMY MEDICAL RESEARCH DETACHMENT
7914 A DRIVE (Bldg 176)
BROOKS AIR FORCE BASE, TEXAS 78235-5138

February 7, 1994

Leave New Orleans on Continental flight #1445 at 6:00 PM, arrive San Antonio on Continental flight #1120 at 8:53 PM.

Hyatt Regency San Antonio
123 Losoya St., San Antonio, TX 78205
Confirmation #HY0000605552

February 8, 1994

8:30 *Overview of USAMRD program*
Bruce Stuck, Director, USAMRD

8:45 *Review of Accidental Laser Exposures and Human Tissue Response*
Donald Gagliano, Commander, USAMRD

9:00 *Overview of LSU Program*
Nicolas G. Bazan, Director, LSU Neuroscience Center

9:10 *The Program: Vision, Laser Eye Injury, and Infectious Diseases*
Herbert Kaufman, Chairman, Ophthalmology Dept. LSU

10:00 *Confocal Approach to Cellular Reactions in Wound Healing and of the Lamina Cibrosa.*
Roger Beuerman of the LSU Neuroscience Center

10:30 **BREAK AND LAB TOUR**

10:50 *Neurochemical Protection of the Brain, Neural Plasticity, and Repair*
Nicolas Bazan, Director, LSU Neuroscience Center

11:40 *Basic Fibroblast Growth Factor (bFGF) Treatment of Laser-Injured Retina*
Steven T. Schuschereba, Chief, Biology Section, USAMRD

12:10 *Role of Growth Factors and Cell Signaling in the Response of Brain and Retina to Injury: Focus on the Retina*
Nicolas Bazan, Director, LSU Neuroscience Center

12:50 **LUNCH**

2:50 Depart San Antonio on Southwest flight #803

5:55 Arrive New Orleans on Southwest flight #1055

LETTERS FROM MEMBERS OF THE EXTERNAL ADVISORY COMMITTEE

WASHINGTON UNIVERSITY SCHOOL OF MEDICINE
AT WASHINGTON UNIVERSITY MEDICAL CENTER

NEUROLOGY

Dennis W. Choi, M.D., Ph.D.

Andrew B. and Gretchen P. Jones Professor and Head
Neurologist-in-Chief, Barnes Hospital

October 17, 1994

Nicholas G. Bazan, MD, PhD
Director, LSU Neuroscience Center
School of Medicine in New Orleans
Louisiana State University Medical Center
2020 Gravier Street, Suite "B"
New Orleans, LA 70112-2234

Dear Nick:

Thank you for the invitation to visit LSU on September 15 and review early progress made under the LSU Neuroscience Center of Excellence Cooperative Agreement with the U.S. Army Medical Research and Development Command.

You have assembled an impressive array of faculty researchers to study diverse aspects of nervous system injury. Overall, I find the individual projects to be thoughtful and well chosen. With you as director, I am sure that they will be most ably integrated. Your project 3 "Neurochemical Protection of the Brain, Neuroplasticity and Repair" is in my view the clear focal point of the overall program. The identification of new PAF antagonist drugs capable of regulating excitatory synaptic transmission and excitotoxic central nervous system injury, is an attractive and attainable goal. The novel pharmacology theme is also well developed in Dr. Moerschbaecher's Section 4 "Neuropharmacology of Delta Receptor Agonist and Antagonist". Involvement of clinician-investigators in clinical departments, such as Dr. Sumner in Project 1 or Dr. Kaufman in Project 5 are strengths of the program that will enhance its ability to identify human therapeutic interventions.

Progress in the first months of operation appears to be on target. Substantial synergy can be expected between the research programs specifically outlined in this collaborative agreement, and the larger intellectual framework formed the LSU Neuroscience Center of Excellence. Your role as director of both efforts is a vital feature that will ensure maximization of this synergy. In summary, I am most enthusiastic about this LSU-U.S. Army Cooperative Agreement, both for its specific merit and as a prototype mechanism for facilitating effective collaboration between academic and military institutions.

Best regards.

Sincerely,

Dennis Choi

Box 8111
660 South Euclid Avenue
St. Louis, Missouri 63110
(314) 362-7175 • FAX (314) 362-2826

525 EAST 68th STREET NEW YORK N Y 10021

THE NEW YORK HOSPITAL-CORNELL MEDICAL CENTER

FRED PLUM, M D , CHAIRMAN
ANNE PARRISH TITZELL PROFESSOR OF NEUROLOGY
CORNELL UNIVERSITY MEDICAL COLLEGE
NEUROLOGIST-IN-CHIEF
THE NEW YORK HOSPITAL- CORNELL MEDICAL CENTER
(212) 746-6141
FAX (212) 746-8532

September 28, 1994

Nicholas G. Bazan, M.D., Ph.D.
LSU Neuroscience Center
2020 Gravier Street
Suite B
New Orleans, LA 70112-2234

Dear Dr. Bazan:

I am pleased to submit this reviewer's report of a Cooperative Agreement between the LSU Neuroscience Center and the US Department of the Army entitled, "Neural Response to Injury: Prevention, Protection and Repair" (henceforth designated as "Injury Study"). The agreement will span four years of effort by the LSU Center; this report describes progress obtained during its first year, extending from September 1, 1993 to August 31, 1994.

Nicholas G. Bazan, M.D., Ph.D. both directs the LSU Neuroscience Center of Excellence and serves as the Program Director of the Injury Study. In addition to Dr. Bazan's personal investigative efforts, seven additional study groups are engaged in research directly related to the Injury Study, as indicated in the administrative diagram attached to this report.

Dr. Bazan's outstanding personal and scientific qualities are the two most important factors in assuring the future success of the LSU-U.S. Army Cooperative Agreement. His leadership and intellectual "taste", as well as his joy in and dedication to brain science penetrate every aspect of the LSU Neuroscience Institute. His enthusiasm has spread to infect his colleagues and many other departments of the Medical School with his high scientific standards and integrity. His knowledge suffuses every dimension of basic neuroscience. His diplomacy and gentle handling of his staff creates their huge loyalty. His energy is contagious. Furthermore, he has the wonderful quality of scientific generosity: always ready to help and encourage others, he is entirely responsible for the continuously improving quality of young persons who are coming to LSU to learn and do important neuroscience.

In addition to the above, Dr. Bazan's specific research is internationally recognized as being of the highest caliber. His personal research contributions to the Injury Study during the past year reflects these high qualities in several ways. They have been published in the most competitively prestigious biomedical research journals. They also add new understandings to both the normal and potentially abnormal effects of the platelet-activating factor (PAF). PAF already is known to be a potent mediator of inflammatory and immune responses. What Bazan and his team now have found is that in low concentrations, PAF transmission may enhance memory and repair mechanisms in brain. Alternately, if released in excessively large concentrations or in combination with certain other molecules, PAF appears capable of causing immune-related tissue damage such as occurs with intense inflammation and/or the induction of genetic prostaglandin synthesis, a step that also may injure brain tissue. This fundamental research emphasizes the complexity and often bidirectional responses that may occur when injury strikes the brain. The results are important and illustrate the difficulties which must be overcome in establishing prevention, protection and repair of brain injuries.

Drs. Bazan and Prescott Deininger have succeeded in developing a series of transgenic mice expressing a dominant mutant of platelet derived growth factor (PDGF). Remarkably enough, the animals thus far have shown no major behavioral alteration under

normal developmental conditions. Their reaction to ischemia, seizures and other circumstances has not yet been tested.

Let me turn now to some of the other, supporting projects: **Drs. R. Benerman, D. Kline and A. Sumner** have made good progress in their studies of neurotrophic factors and other mechanisms in human and experimental neuromas resulting from blunt and crush nerve injuries. Basic fibroblast growth factor (bFGF) was the most prominent factor found in human post-nerve injury neuromas with other specific factors either absent or reaching only very low levels of concentration. More precisely analytic experiments await the analyses of fresh neuronal material from the experimental preparations.

Drs. Herbert Kaufman and Roger Benerman have made brilliant advances using confocal microscopy to examine the cellular details of the human retina. To a degree never before possible they have safely demonstrated in awake human subjects the acute pathophysiology of laser injuries to cornea and their early transformation into fibroblasts. Detailed identification of anterior chamber cells has been possible and current efforts are underway to examine at great magnification the optic disc itself. Ocular fungus and herpes infections can be identified immediately and without introducing foreign substances against the cornea or into the eye. Application of the tool should have an important place in clinically applied military medicine.

During the past year, the investigators also have pursued their earlier discovery that ambient chilling of monkeys latently infected with H. Simplex induces an acute recurrence of cutaneous herpes. Furthermore, chronic ingestion of the beta blocker, propanelol, has been found to ameliorate or prevent the active recurrence. Clinical trials of this important discovery must be pursued as it has important practical aspects.

During the year, the necessary work to establish and equip the glaucoma research laboratory was undertaken. Next year's report can be expected to provide research results from that laboratory.

Dr. Joseph Moerschbaecher and his colleagues in pharmacology have initiated preliminary studies on the influence of delta opoid agonists-antagonists on learning and antinociception. Somewhat surprisingly, the agent damps the CO_2 response of breathing but has no antinociceptive effect. The same investigator is analyzing how anxiogenic drugs affect dopamine neurons in the ventral tegmental area of the rodent brain.

In another preliminary approach, **Drs. H.W. Thompson et al** have initiated experiments passing retroviral gene carriers into the eye with externally applied eye drops, thereby developing a new approach to deliver protection against certain ophthalmologic infections or enhancing the potential success of corneal transplant.

Drs. Richard Bobbin and Charles Berlin, thanks to the DOD grant, have added an excellent postdoctoral student as well as important new equipment to their laboratory. The laboratory's principal subject of interest is to find mechanisms for preventing the audiologic damage produced by intense sound. In guinea pigs, this has been achieved by stimulating calcium-dependent mechanisms in cochlear neurons. In another study, the laboratory has found in human studies that during the delivery of loud, binaural sounds, men and women suppress the noise in opposite sided ears from each other.

The above individual achievements provide only a part of the considerable effort, enthusiasm and success that the U.S. Army grant has brought to the LSU Neuroscience Center of Excellence (NCE). The following steps forward can also be emphasized:

1) Morale in the LSU-NCE rides at high pitch, encouraging scientific collaboration and the generation of new ideas.

2) Funds have been granted to subsidize the necessary equipment and technical personnel to establish a brain bank. Presently, approximately 50 specimens are available in storage with the Center holding good clinical records of the preterminal illness.

3) A program of "starter" grants designed to assist young investigators in conducting merit-deserving, self designed research projects has been initiated.

4) A highly popular state-wide Graduate School outreach summer program has been successfully concluded, attracting a strong interest in neuroscience among gifted college students.

5) An interdisciplinary graduate program in neuroscience was initiated and strongly encouraged by the faculty during 1993-94. As a result, nearly all of the graduate students (including the new entering class) are of very good quality. Indeed, other participating departments say that the Neuroscience graduate students are the best among the LSU biological sciences programs.

Summary. Under the generous auspices of a U.S. Army Cooperative Agreement, the LSU Neuroscience Center of Excellence is not only thriving but headed for far greater future productivity than at any time in the past. The admirable success of the program depends heavily on the foresight, intelligence, creativity and energy of two outstanding scientists, Herbert Kaufman and, especially, Nicholas G. Bazan. Their achievements and those of their colleagues totally warrant continuation of support. Indeed, every indication is that their extramural, non-Army support will continue to grow, making the program stronger and stronger as the years elapse.

One serious problem remains - that of sufficient space in which to do the studies that Dr. Bazan and his colleagues already have conceived so well. Prompt attention to and effective application of must be given to the DOD funds already awarded to construct new research space which will greatly increase the LSU Neuroscience team's opportunities for creative discovery.

I and my colleagues on the External Advisory Board of the LSU Neuroscience Center of Excellence strongly endorse the quality and number of achievements that have come from the U.S. Army-LSU-NCE collaboration. Thanks to strong leadership for the Center and a high degree of internally high morale and interdependence within the Center, it can be anticipated that the Cooperative Agreement will have a major impact on national neuroscience research as well as the specific medical needs of the U.S. Army.

Sincerely,

Fred Plum

Fred Plum, M.D.

FP/moc

NEUROPHARMACOLOGY OF DELTA RECEPTOR AGONSITS AND ANTAGONISTS

Project Director:

Joseph Moerschbaecher, Ph.D.

Participating Scientists:

Charles France,PhD
Dennis J. Paul, PhD
Jayaraman Rao, MD

FRONT COVER

COOPERATIVE AGREEMENT NO.: DAMD17-93-V-3013

TITLE: Neural Responses to Injury: Prevention, Protection, and Repair

CHAPTER: Neuropharmacology of Delta Receptor Agonists and Antagonists

INVESTIGATOR: Joseph Moerschbaecher, Ph.D., Principal Investigator
Department of Pharmacology, LSU Medical Center
INVESTIGATOR ADDRESS: 1901 Perdido, New Orleans, LA

REPORT DATE: 1 August, 1994

TYPE OF REPORT: Annual Report - 20 September, 1993, through July, 1994

PREPARED FOR:

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Program Project Director
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FOREWARD

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( ) In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources Commission on Life Science, National Research Council (NIH Publication no. 86-23, Revised 1985).

( ) For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45 CFR 46.

( ) In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

Investigator Signature

Charles P. France (Co-PI) for J.M. Moerschbaecher

ANIMAL USE
20 SEPTEMBER, 1993, THROUGH JULY, 1994

DAMD17-93-V-3013

The experimental animals used during this period for the project, Neural Responses to Injury: Prevention, Protection, and Repair, **Subproject: Neuropharmacology of Delta Receptor Agonists and Antagonists**, are as follows:

Species	Number Allowed	Number Used	LSU IACUC #
rhesus monkey	6	6	1062

Investigator Signature

Charles P. France (Co-PI) for
J.M. Moerschbaecher

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ABSTRACT

Studies in the Division of Neuropharmacology are investigating the role of endogenous opioid systems in learning and memory, ventilatory function and antinociception. The goal of these studies is: to identify and characterize candidate ligands that might be useful in studies on *delta* opioid mechanisms; and to use these compounds to systematically investigate the role of *delta* systems in complex behavioral processes, in ventilation and in the perception of noxious stimuli. The first candidate compound is BW373U86, which has been suggested to be a highly-selective agonist for the *delta* opioid receptor. In preliminary studies, BW373U86 has effects that are unlike effects obtained with prototypic *mu* or *kappa* opioid agonists. BW373U86 disrupts learning at doses that have no significant effect on performance of a previously-acquired task; in contrast, *mu* and *kappa* agonists disrupt learning and performance at the same doses. Whereas *mu* agonists have pronounced respiratory-depressant effects that result from decreases in frequency and volume of ventilation, BW373U86 appears to have biphasic effects on frequency and volume of ventilation that can be dissociated temporally. BW373U86 not only diminishes hyperventilation that occurs in the presence of CO_2, there is a suggestion that overall ventilatory response might be reduced by CO_2. Finally, despite clear demonstrations of behavioral effects under other conditions that do not appear to be attributable to actions at *mu* or *kappa* opioid receptors, BW373U86 is without effect in studies of antinociception. Together with recently-published data on BW373U86, results of the current studies provide support for the potential utility of this compound in future studies on *delta* opioid systems.

INTRODUCTION

Endogenous opioid systems are thought to be important in the modulation of various appetitive behaviors, including food and water consumption; however, the role of these endogenous systems in other physiological and behavioral conditions is not well established despite many years of research on opioid receptors and opioid receptor ligands. Though much is known about the neuroanatomy, neurochemistry and neurophysiology of opioid systems in brain, considerably more is known about *mu* and *kappa* opioid systems as compared to *delta* opioid systems. This discrepancy is not due to any apparent lack of importance of *delta* opioid systems, but rather to a paucity of appropriate compounds for conducting thorough evaluations of *delta* opioid systems. Unlike *mu* and *kappa* opioid systems, where a multitude of systemically-active and receptor-selective agonists and antagonists are available, until recently the only available *delta*-selective compounds were peptides unsuitable for many types of studies.

Recently, a series of papers was published describing the effects of a systemically-active (alkaloid), *delta*-receptor selective agonist under a variety of conditions *in vitro* and *in vivo*, in mice, rats, pigeons, squirrel monkeys, and rhesus monkeys (Chang et al., 1993; Comer et al., 1993a,b; Dykstra et al., 1993; Lee et al., 1993; Wild et al., 1993). Included among these extensive studies is the first demonstration of an effect of BW373U86 in rhesus monkeys that appears to be mediated by *delta* opioid receptors (Negus et al., 1993). In monkeys responding on a lever under a schedule of food presentation, BW373U86, like virtually every biologically-active drug, disrupted responding in a dose-related manner; however, the selectivity of BW373U86 was

demonstrated by the potency of the selective opioid antagonist quadazocine in attenuating this behavioral effect of BW373U86. The importance of this finding is not only that a behavioral effect attributable to *delta* receptor actions was evident in rhesus monkeys, but also that parenterally-administered BW373U86 clearly had effects that were likely mediated in the central nervous system. The feasibility of the studies proposed in the current research effort is significantly enhanced by the availability of this systemically-active *delta*-selective agonist.

The specific hypotheses that are being evaluated by the research conducted in the Division of Neuropharmacology, and with support of the current agreement, are that *delta* opioid systems play a tonically active role in modulating: 1) learning and memory; 2) ventilatory function; and 3) antinociception. If these hypotheses are supported, endogenous *delta* opioid receptor systems would represent one neurochemical system that might be susceptible to both positive and negative modulation, thereby providing a potential pharmacotherapeutic approach for the enhancement of behavioral and physiological performance.

METHODS AND RESULTS

A portion of the first budget year was devoted to the procurement of experimental equipment, supplies and subjects. Immediately upon confirmation of funding for this research component, bids were solicited for the purchase of six rhesus monkeys. Shortly after the bids were received a purchase order was issued and within several months the monkeys arrived at LSU Medical Center where they were

quarantined for 90 days in the Division of Animal Care at the LSU School of Dentistry. During this time equipment and supplies required for these studies were manufactured or purchased. Upon completion of the quarantine period, two monkeys were transported to the Medical Education Building for use in studies on learning and memory with the other four monkeys remaining at the School of Dentistry.

Procedures used to evaluate the effects of drugs on learning and memory have been used extensively in the laboratory of the Principal Investigator and full details of these procedures are available elsewhere (Moerschbaecher et al., 1983, 1985, 1987; also see original application). In regards to studies of the effects of *delta* opioid agonists and antagonists on learning and memory we have completed one preliminary study and are nearing completion of a larger study in monkeys as was described in our original proposal. Each of these studies are briefly described below.

In our preliminary study, which was in progress at the time our original proposal was submitted, responding by squirrel monkeys was maintained by food presentation under a repeated acquisition of behavioral chains procedure. Subjects were required to acquire a different three-response chain each session. Sequence completions were reinforced under a fixed-ratio 5 schedule, while errors produced a brief timeout. The *mu* opioid agonist morphine (0.1 - 0.56 mg/kg) produced dose-related decreases in response rate at doses which had no effect on percent errors during acquisition. The *kappa* opioid agonist, U-50,488 (0.018 - 0.56 mg/kg) produced a steep dose-effect curve decreasing response rate only at the larger doses while having little or no effect on acquisition. In contrast, the *delta* opioid agonist BW373U86 (0.0056 - 0.32 mg/kg)

produced dose-related decreases in response rate and increases in errors during acquisition. The *delta* opioid receptor antagonist naltrindole (0.056 - 18 mg/kg) had no effect on either rate of responding or percent errors when administered alone. However, naltrindole antagonized the disruptive effects of BW373U86 on acquisition at doses that failed to antagonize the rate-decreasing effects of morphine or U-50,488. These data further support our initial hypothesis that, in contrast to *mu* and *kappa* opioid receptors, the *delta* receptor plays an important role in the learning process. This preliminary study has been submitted for publication to the Journal of Pharmacology and Experimental Therapeutics.

In a second study we have begun a systematic investigation of the effects of BW373U86 on complex behavioral processes in rhesus monkeys. In this study, responding was maintained under a multiple schedule of repeated acquisition and performance of conditional discriminations. Under this procedure, in one component of the multiple schedule monkeys were required to learn a new discrimination each session, while in the other component the discrimination was the same each session. Using this procedure we are able to evaluate the effects of a drug on both the learning and the performance of discrimination in a single session. Control and drug sessions are shown for a single monkey in the cumulative records of Figure 1, Appendix A. In these records, correct responses stepped the pen upward and reinforcer delivery deflected this same pen downward. The lower event pen was deflected downward each time an incorrect response (error) was made. The stepping pen also reset and the event pen deflected each time the components changed. A cumulative record for a control session

is shown in the top panel of Figure 1, Appendix A. Note that under control conditions, errors (event pen) decrease within the first acquisition component and that no errors are made in performance. Behavior in each of the components quickly becomes comparable. The effects of 0.1 mg/kg of BW373U86 on responding in this same subject are shown in the middle panel of Figure 1, Appendix A. Note that BW373U86 produced a large error-increasing effect in acquisition while having little or no effect on responding in the performance component. At this dose, BW373U86 retarded acquisition until near the end of the session as is indicated by the arrow. The effects of 0.32 mg/kg of BW373U86 are shown in the lower panel of Figure 1, Appendix A. Note that at this dose, acquisition was blocked; there were few correct responses and a large number of errors. In contrast, in the performance component the disruption in responding was much less, with only a short pause in responding and a few errors indicated by the arrows.

Dose-effect curves for BW373U86 are shown for two monkeys in Figure 2, Appendix A. Note that this purported *delta* agonist increased percent errors and decreased response rate at doses which had little or no effect on performance. These data further support our hypothesis that the *delta* opioid system plays an important role in learning.

Finally, we have recently begun studies that are designed to characterize the effects of the *delta* antagonist naltrindole on a multiple-schedule procedure of learning and performance. Specifically, we are interested in determining the effects of naltrindole alone and in combination with BW373U86. These experiments should help

determine both the specificity of this system and its level of tonic activity. These and additional studies should be concluded during the next project year.

A procedure has been developed for the study of respiratory effects in unanesthetized rhesus monkeys (Butelman et al., 1993; France and Woods, 1990; Howell et al., 1988). While incorporating several design changes from the original experimental configuration for studying respiration in monkeys, a fully-operational apparatus has been developed during the current budget year. Briefly, monkeys are trained to sit quietly in chairs that provide restraint at the neck and waist. A head plethysmograph (helmet) is placed over the head of the subject and secured to the top of the chair by a series of rubber dams and plastic harnesses. The configuration of dams and plastic is such that an air-tight seal is maintained around the neck of the monkey. Air, or 5% CO_2 in air, is delivered (rate = 10 liter/minute) to the helmet from tanks via flow valves and is removed by a vacuum pump. A pressure transducer is connected to the helmet and detects changes in pressure that are reflective of inspiration and expiration. During experimental sessions monkeys are seated in a sound-attenuating chamber. Adjacent to the chamber is a Grass polygraph and a computer which are used to measure ventilatory responses and to store data. Pressure changes are measured and stored continuously throughout experimental sessions. All drugs are administered s.c. in the back in a volume of 0.1 ml/kg body weight.

The nature of the experimental apparatus used in studies of respiratory function necessitates that monkeys be introduced gradually to the study in a controlled,

systematic manner. In order to obtain reliable measures of ventilatory function, monkeys must sit relatively still during the experiment; thus, a period of at least several weeks is required to acclimate monkeys to the chair, the helmet and the 10 liter/minute flow of gas through the helmet. To date, four monkeys have been adapted to the experimental conditions and two monkeys have been tested with drugs.

Prior to the initiation of studies on *delta* systems and their role in ventilatory function, control studies were conducted with known physiologic (air and 5% CO_2 in air) and pharmacologic (morphine) conditions. These preliminary studies allowed us to validate our experimental apparatus by comparing results obtained in these studies to historical controls determined with other monkeys at other institutions (e.g., Butelman et al., 1993, France and Woods, 1990).

Figure 1, Appendix B, shows minute volume (V_E, upper), tidal volume (V_T, middle) and frequency (f, lower) of ventilatory responses in monkey GO breathing air (open symbols) or 5% CO_2 in air (closed symbols). Exposure to CO_2 produces an marked increase in f, a modest decrease in V_T, and an overall increase in V_E. The increase in ventilation produced by 5% CO_2 is no longer evident within six minutes of the termination of CO_2 exposure. This rapid recovery of ventilation after exposure to CO_2 allows for repeated testing of drug effects, in air and in CO_2, within the same experimental session. Figure 2, Appendix B, shows a more detailed analysis of three seven-minute exposures to 5% CO_2, each separated by 30 minutes of breathing in air, in two monkeys. For monkey GO (left) and monkey MA (right), 5% CO_2 increases V_E to 140-180 percent of control; these increases are due largely to increases in f with little

change in V_T for monkey GO and to increases in V_T with very modest increases in f in monkey MA. These results are consistent with the temporal relations described previously for CO_2-induced hyperventilation in rhesus monkeys and will provide the basis for studies on the effects of drugs in monkeys breathing air or CO_2.

The effects of morphine on ventilatory function were studied initially in monkeys breathing air. First, single doses of morphine were administered and ventilation was monitored for a three-hour period. In other studies, increasing doses of morphine were administered every 30 minutes using a multiple-dosing procedure whereby the cumulative dose increases by 0.5 log units per injection (e.g., 0.1 mg/kg [first injection] plus 0.22 mg/kg [second injection] yielding a second cumulative dose of 0.32 mg/kg). Figure 3, Appendix B, shows two dose-effect determinations for morphine, determined on different days, in monkey MA. In this monkey and another (data not shown), morphine decreases ventilation in air in a dose-related manner with a dose of 3.2 mg/kg producing an overall decrease of 50-60% in V_E (upper panel). These results are consistent with published data on the effects of morphine on ventilation in air in rhesus monkeys.

Having demonstrated reliable changes in ventilation in the presence of 5% CO_2 and having determined appropriate dose-effect curves for the effects of morphine on ventilation in air, recent studies have begun to examine the effects of the purported *delta*-receptor selective agonist BW373U86 on ventilation in rhesus monkeys. Figure 4, Appendix B, shows a time-course study for the effects of a single injection of 3.2 mg/kg of BW373U86 in monkey GO breathing air. In the first hour after administration of

this dose of BW373U86, there is a 30% decrease in V_T (middle) and a slight increase in f (lower), the combined effect of which is a 20% decrease in V_E (upper). Though the effect on V_T wanes by 70 minutes post-injection, at the same time f decreases so that V_E remains decreased over the next two hours. These preliminary results suggest the effects of BW373U86 on ventilation might involve multiple actions, perhaps as a result of interactions with endogenous opioids that might be mobilized during periods of compromised ventilation, and that complete time- and dose-effect analyses will need to be conducted for a variety of doses in order to fully characterize these complex effects.

Preliminary studies have also been conducted in which the effects of BW373U86 have been assessed in a monkey exposed to 5% CO_2 for seven minutes once every 30 minutes. Figure 5, Appendix B, shows the effects of BW373U86 on CO_2-induced hyperventilation; whereas in the absence of drug, ventilation is increased significantly in GO within 4 minutes of exposure to 5% CO_2, in the presence of 5.6 mg/kg of BW373U86, there is a slight decrease in V_E (upper) during the first two minutes of CO_2 exposure and an apparent delay in the hyperventilation typically observed in the presence of CO_2 (compare left panels, Figure 2 to Figure 5, Appendix B). Like the biphasic effects observed with BW373U86 in monkeys breathing air (Figure 4), this unusual ventilatory response suggests the effects of this compounds are not due simply to a direct agonist action at a homogenous pool of receptors.

The procedure used to study antinociceptive effects of drugs has been described in detail elsewhere (Dykstra and Woods, 1986; France et al., 1989, 1994). Monkeys are

seated in primate chairs that provide restraint at the neck. The lower 10-12 cm of the shaved portion of the tail is immersed in a thermos containing water of 40, 50 or 55° C. and the time for monkeys to remove their tails from the thermos is measured and recorded with a hand-held switch connected to a microprocessor.

To date, the antinociceptive effects of other prototypic opioids (*mu* and *kappa* agonists) have been studied preliminarily in rhesus monkeys and a single study has been conducted with the *delta*-selective agonist BW373U86. Up to a dose of 56.0 mg/kg s.c., BW373U86 failed to increase significantly the latency for monkeys to remove their tails from a thermos containing 50 or 55° C. water. Emesis occurred in one monkey within several minutes of the administration of 56.0 mg/kg.

CONCLUSIONS

The initial goal of these studies was to evaluate candidate compounds for their utility in subsequent studies on the role of *delta* opioid systems in selected behavioral and physiological events. Under all three experimental conditions, the purported *delta*-selective agonist BW373U86 has been shown to have behavioral effects that are not identical to the effects obtained with either *mu*- or *kappa*-selective agonists under the same experimental conditions. The pattern of effects observed in studies on learning and memory and in studies on respiratory function, clearly distinguish BW373U86 from prototypic *mu* (e.g., morphine) and *kappa* (e.g., U-50,488) opioids. The lack of effect in studies of antinociception was unexpected and could result from several different factors, as discussed below. Nevertheless, these preliminary results strongly support the

premise of these studies that *delta* opioid systems are important in the mediation of behavioral and physiological effects and that these behavioral and physiological effects might be amenable to modification (e.g., enhanced cognitive function) by compounds with actions at *delta* receptors or by compounds that otherwise modify endogenous opioid systems.

The effects observed with BW373U86 in studies of respiration, though preliminary, are compelling. One possibility regarding *delta* opioid systems and ventilation, though entirely untested by these data, is that CO_2-induced hyperventilation is mediated, at least in part, by endogenous opioids, the levels of which are modified acutely by the administration of exogenous *delta* agonists. This is a specific hypothesis which we will evaluate further, first by examining the susceptibility of these effects to antagonism by the *delta*-selective antagonist naltrindole and then by studying whether the effects of BW373U86 on ventilation are modified by peptidase inhibitors that should enhance any actions that are mediated by endogenous opioid peptides.

The negative data obtained in the antinociception study suggest several different interpretations. First, it is possible that the BW373U86 does not readily access the central nervous system in rhesus monkeys and, therefore, that the positive results obtained previously with BW373U86 in rhesus monkeys were due to peripheral actions of this compound. This interpretation appears unlikely in light of the numerous demonstrations of what appear to be centrally-mediated behavioral effects (e.g., discriminative stimulus effects, convulsions) in other species as well as the preliminary data discussed above for the effects of BW373U86 on learning and memory and on

ventilation in rhesus monkeys. A second interpretation is that BW373U86 has such limited efficacy at *delta* receptors that it can not produce antinociceptive effects under these experimental conditions. This interpretation is not supported by a considerable amount of evidence in other species that suggests BW373U86 has relatively high efficacy at *delta* opioid receptors. Finally, it is possible that *delta* opioid receptors do not mediate antinociceptive effects in rhesus monkeys. While there have been many demonstrations of *delta*-receptor mediated antinociceptive effects for BW373U86 as well as other *delta*-selective ligands in other species, there have been no demonstrations of antinociceptive effects in rhesus monkeys where the relevant receptor has been clearly shown to be *delta* opioid. While the paucity of systemically-active *delta* agonists has prevented further research in this area, it is hoped that other compounds, including compounds in the BW373U86 series, can be used to further evaluate the role of *delta* receptors in antinociception in non-human primates.

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APPENDICES

Appendix A:

Figure 1. Cumulative records for control, 0.1 mg/kg and 0.32 mg/kg of BW373U86.

Figure 2. Dose-effect curves for BW373U86 on learning and performance.

Appendix B:

Figure 1. Ventilation in air (open) and in 5% CO_2 in air (closed) in monkey GO.

Figure 2. Effects of exposure to 5% CO_2 on ventilatory function in monkeys GO and MA.

Figure 3. Effects of morphine on ventilation in normal air in monkey MA.

Figure 4. Time-course study of the effects of 3.2 mg/kg of BW373U86 on ventilation in air in monkey GO.

Figure 5. Effects of 5.6 mg/kg of BW373U86 on ventilation in 5% CO_2 in monkey GO.

APPENDIX A

Figures 1 and 2

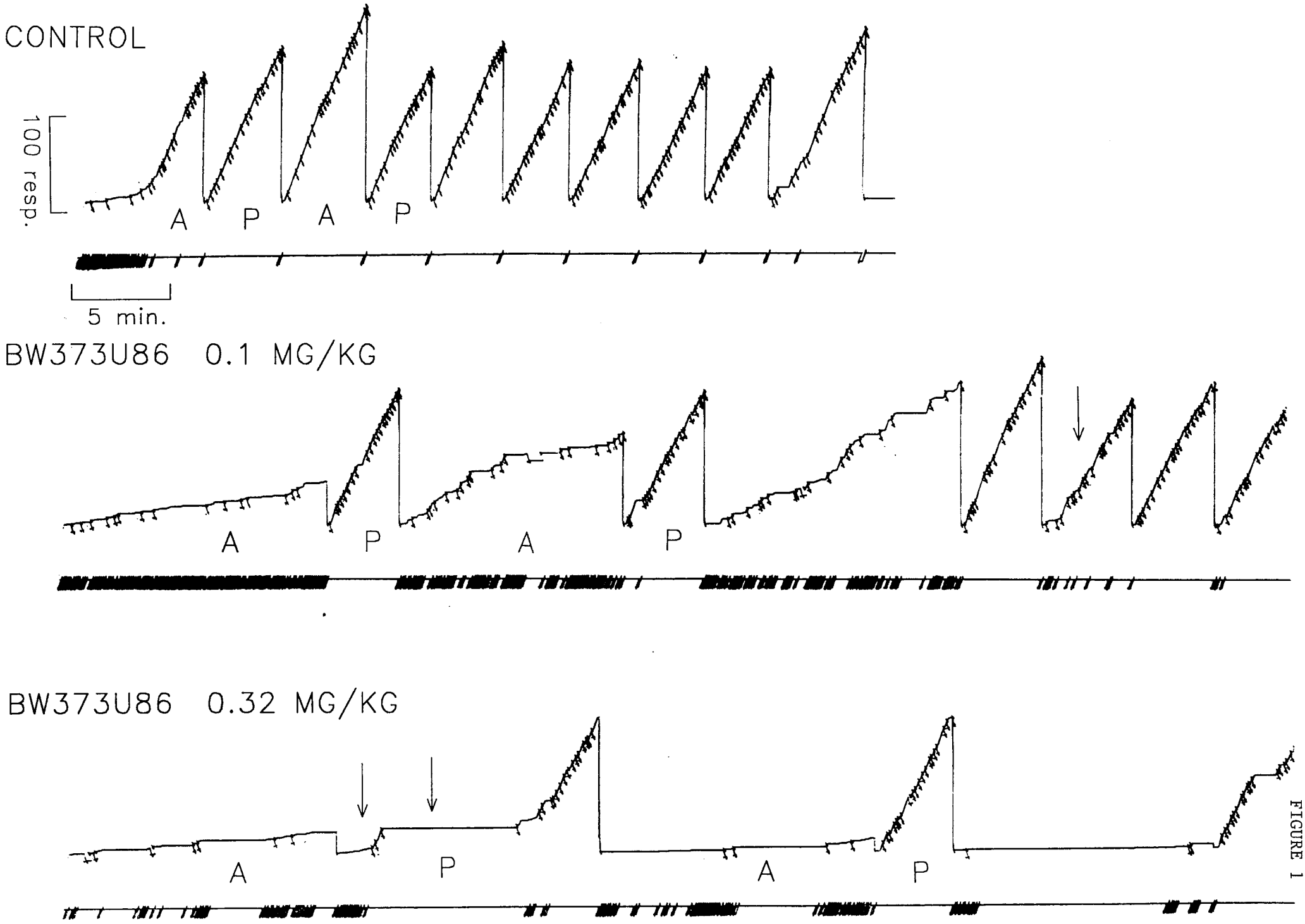
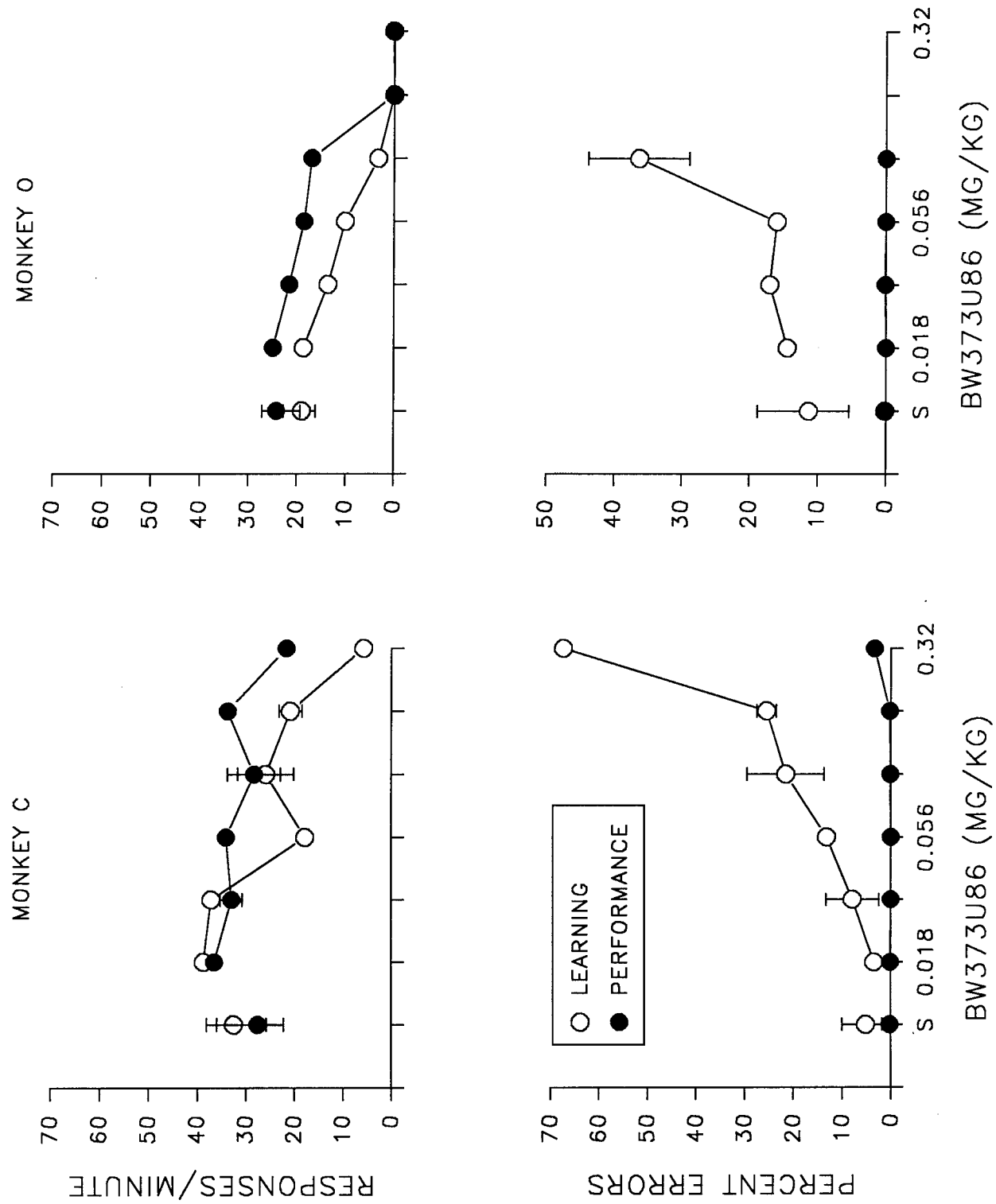

CONTROL
100 resp.
A
P
A
P
5 min.
BW373U86 0.1 MG/KG
A
P
A
P
BW373U86 0.32 MG/KG
A
P
A
P

FIGURE 1

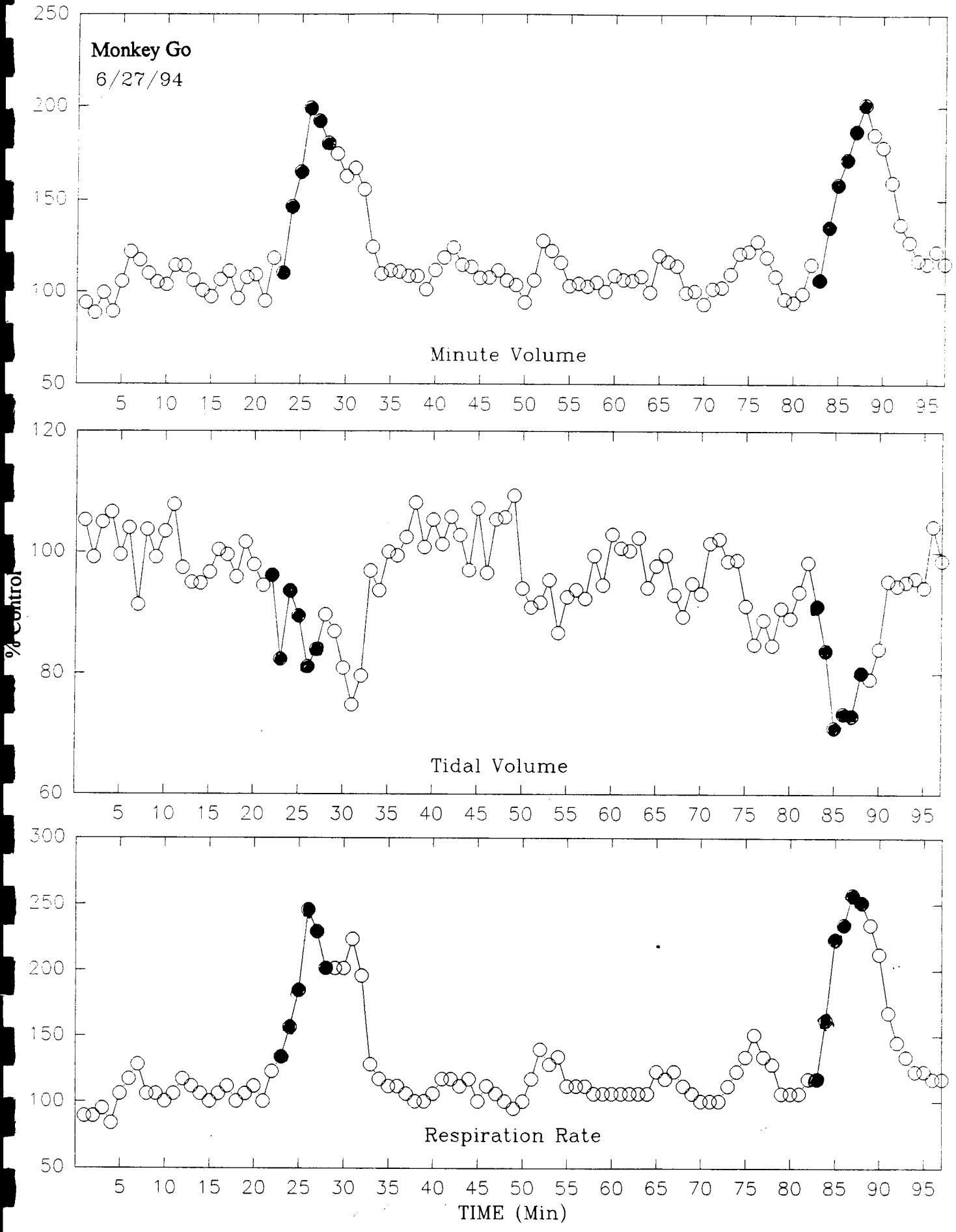
FIGURE 2

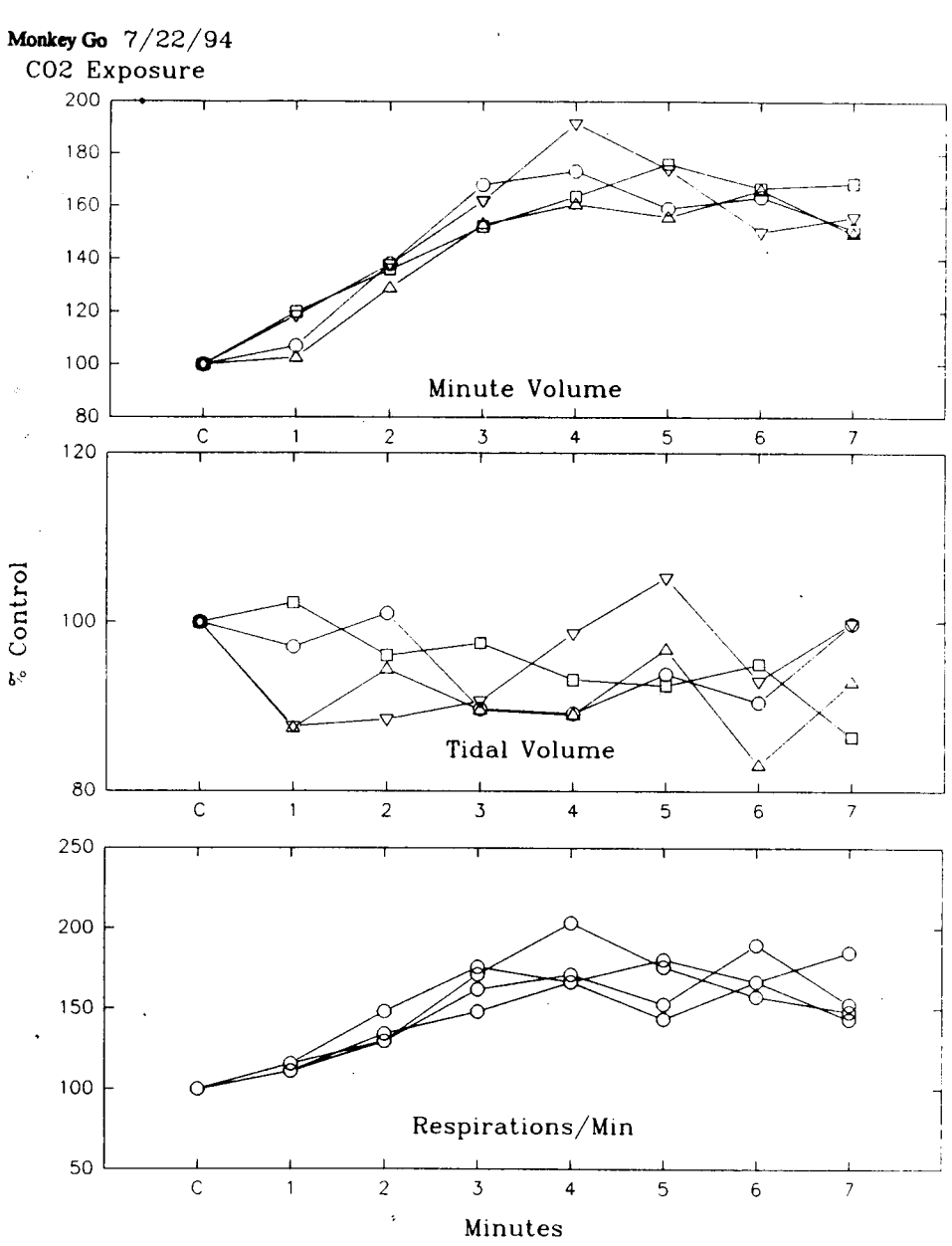

MONKEY C
MONKEY O
RESPONSES/MINUTE
PERCENT ERRORS
LEARNING
PERFORMANCE
S 0.018 0.056 0.32
BW373U86 (MG/KG)
0
10
20
30
40
50
60
70

APPENDIX B

Figures 1, 2, 3, 4 and 5

FIGURE 1


Monkey Go
6/27/94
Minute Volume
Tidal Volume
Respiration Rate
% Control
TIME (Min)


Monkey Go 7/22/94
CO2 Exposure
Minute Volume
Tidal Volume
Respirations/Min
% Control
Minutes
C 1 2 3 4 5 6 7

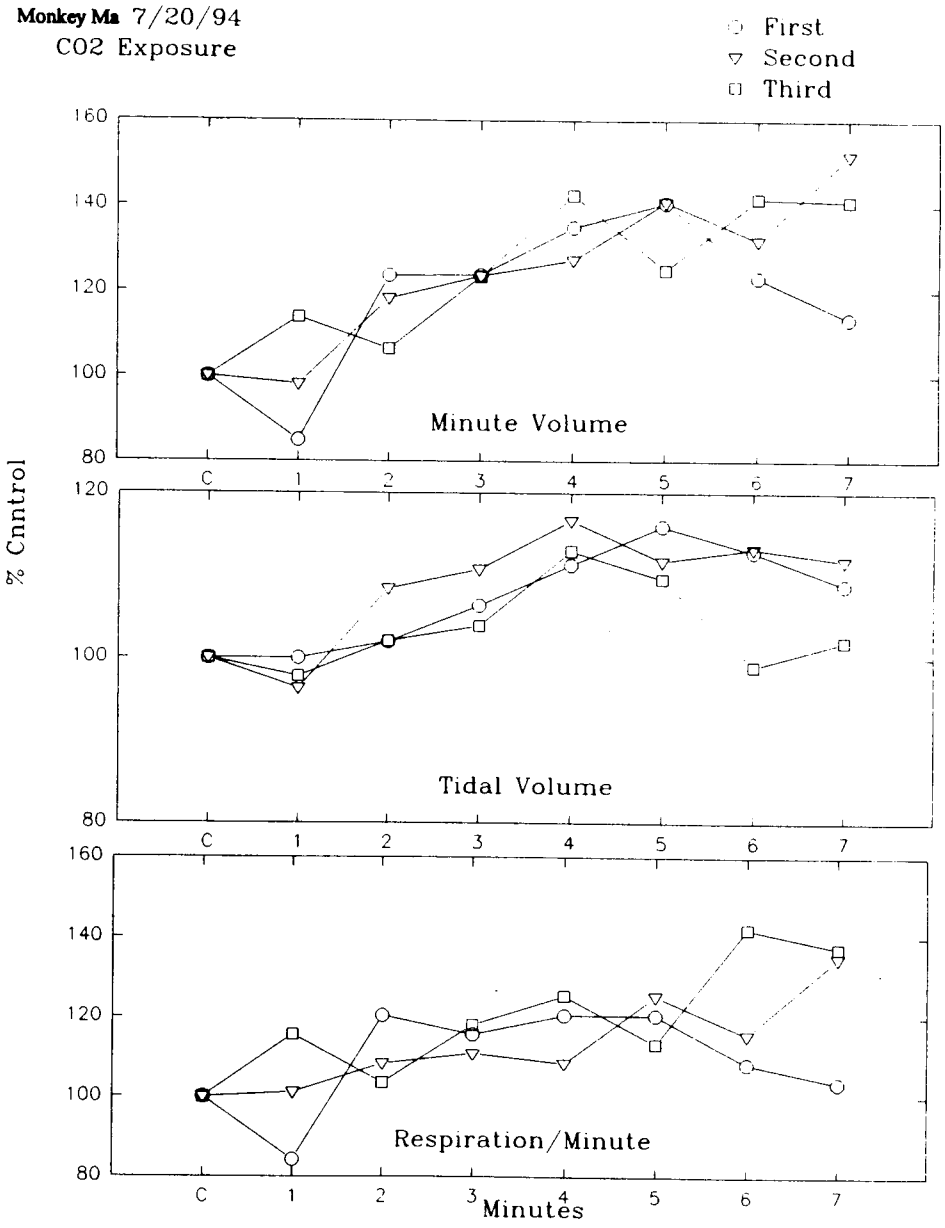
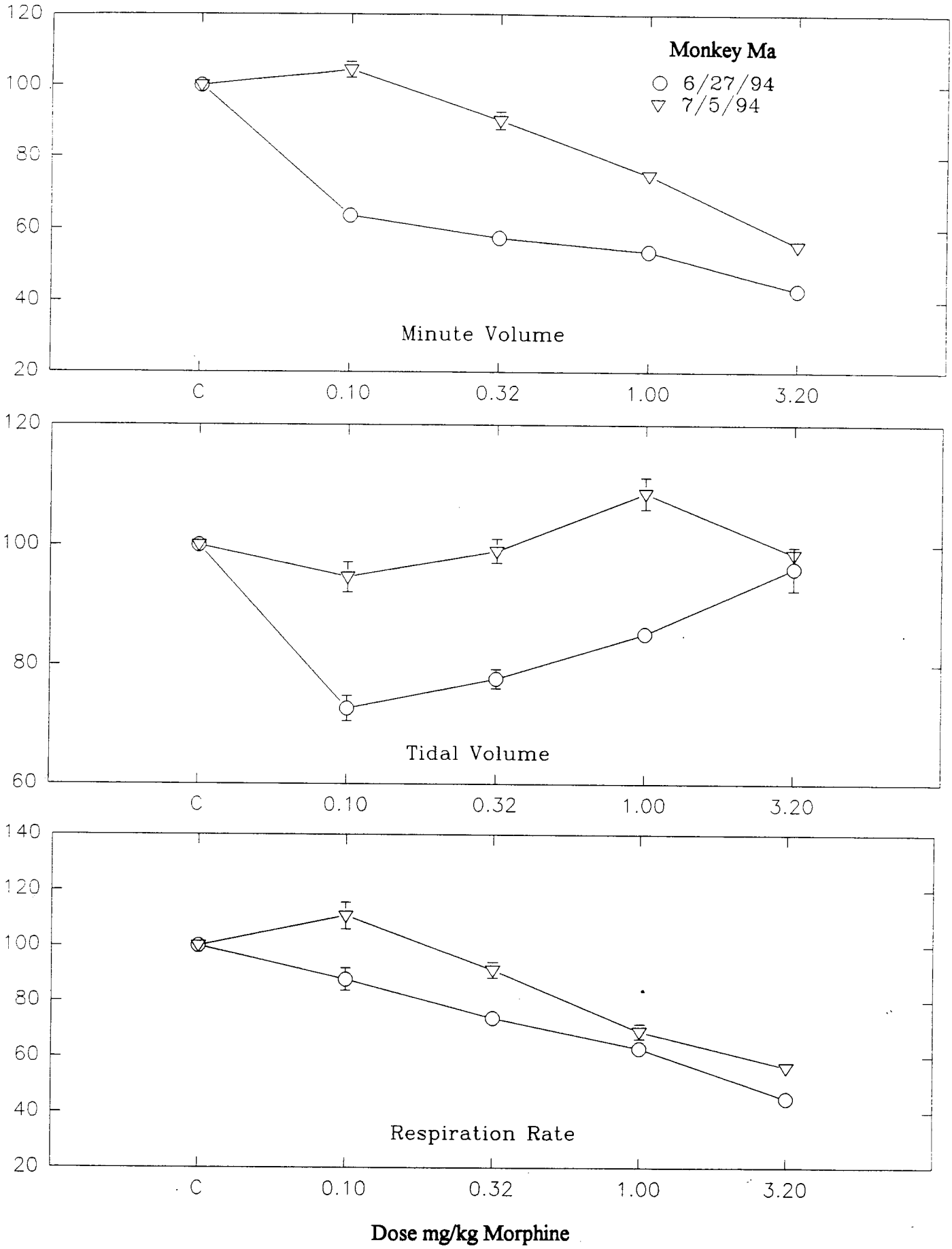
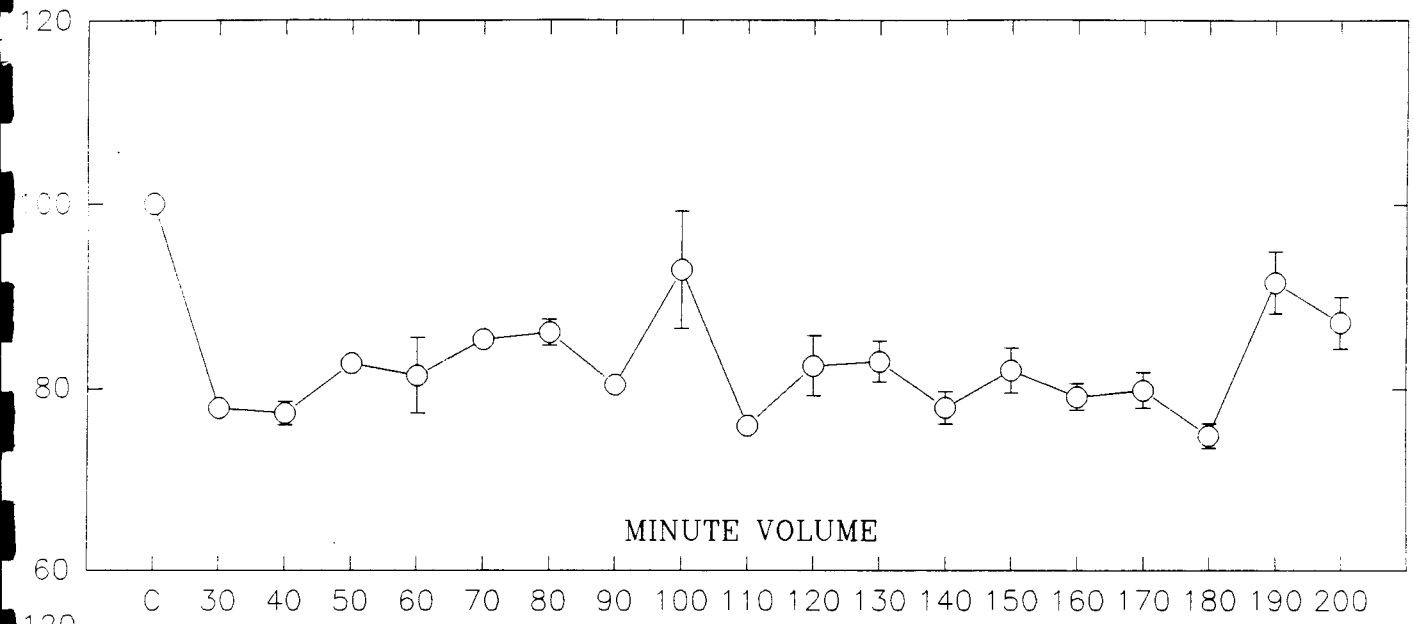

Monkey Ma 7/20/94
CO2 Exposure
First
Second
Third
Minute Volume
Tidal Volume
Respiration/Minute
% Cntrol
Minutes
C 1 2 3 4 5 6 7

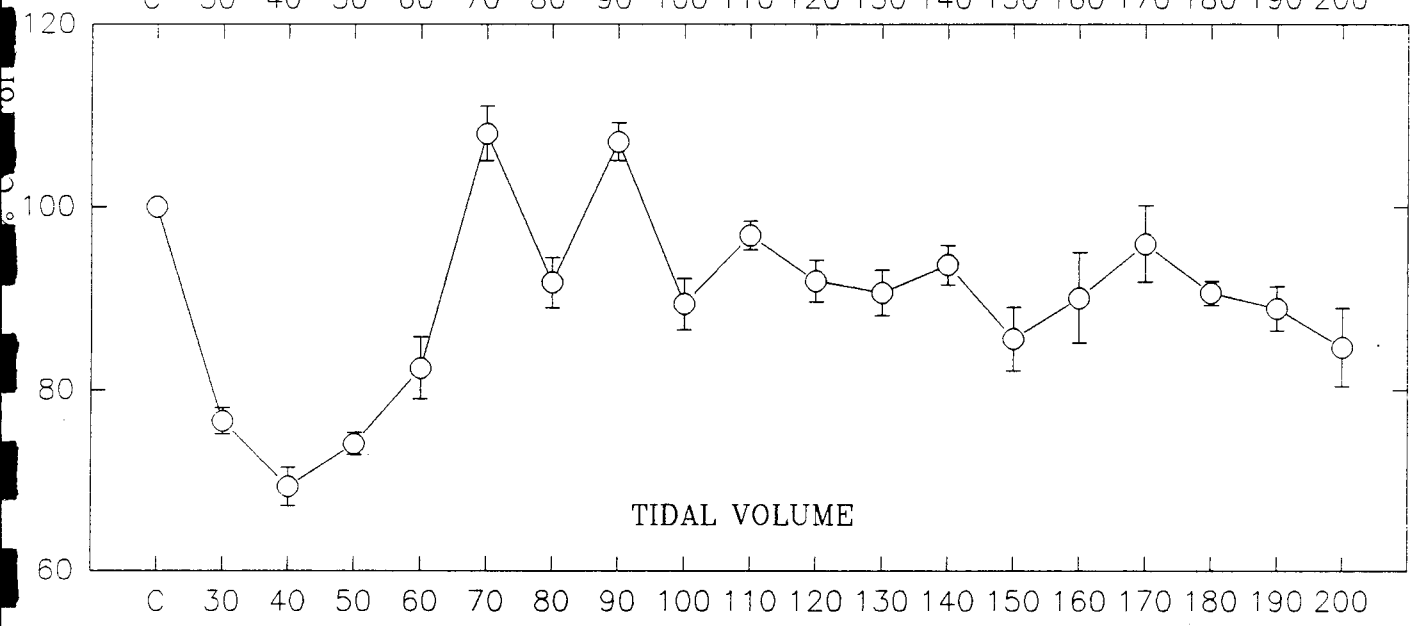
FIGURE 2


Monkey Ma
6/27/94
7/5/94
Minute Volume
Tidal Volume
Respiration Rate
% Control
C
0.10
0.32
1.00
3.20
Dose mg/kg Morphine

3.2 mg/kg BW373U86

Monkey Go





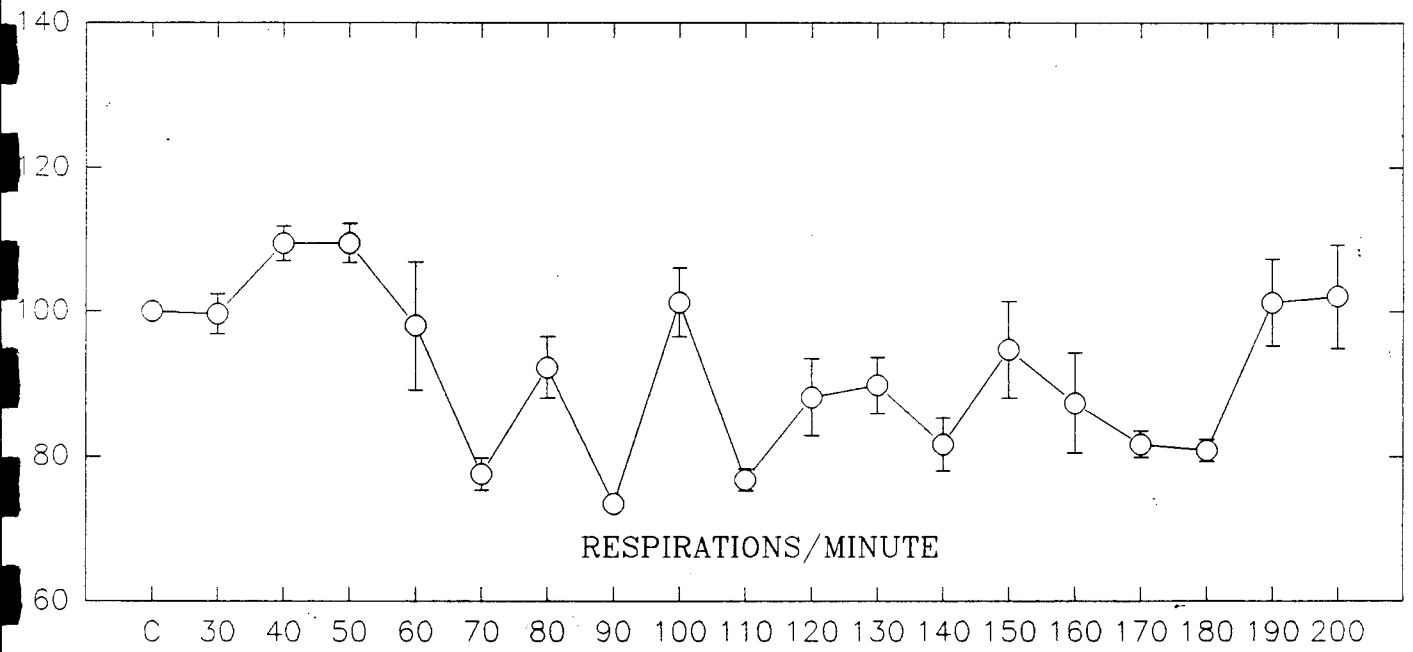
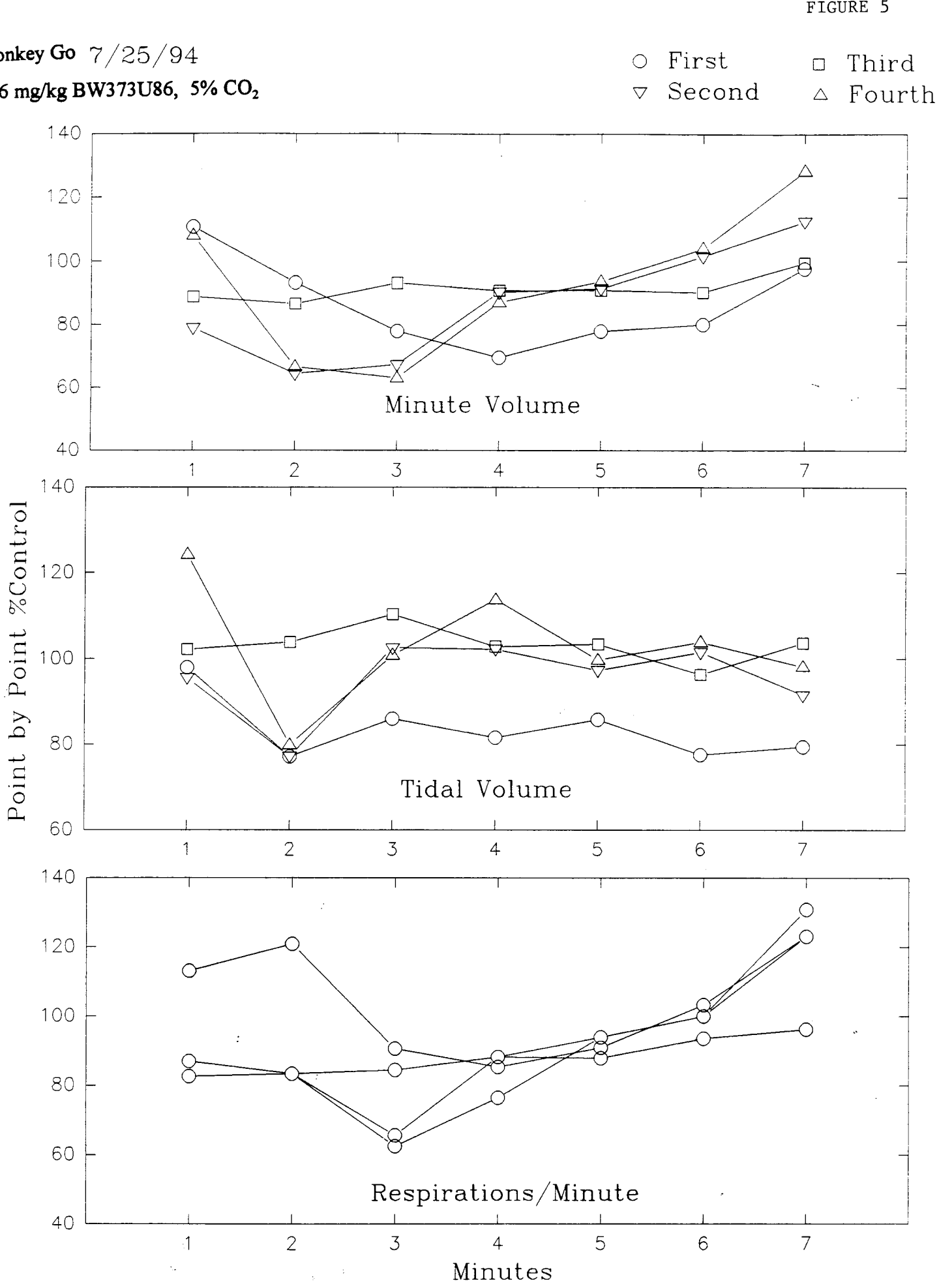


FIGURE 5

Monkey Go 7/25/94

5.6 mg/kg BW373U86, 5% CO_2



NEUROPHARMACOLOGY OF DELTA RECEPTOR AGONISTS AND ANTAGONISTS

Subproject: Dopamine, and Opiate Receptors

Project Director:

Joseph Moerschbaecher, Ph.D.

Subproject Director:

Jayaraman Rao, MD

FOREWARD

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(✓) For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45 CFR 46.

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Investigator Signature

ANIMAL USE
20 SEPTEMBER, 1993, THROUGH JULY, 1994

DAMD17-93-V-3013

The experimental animals used during this period for the project, Neural Responses to Injury: Prevention, Protection, and Repair, **Subproject: Neuropharmacology of Delta Receptor Agonists and Antagonists**, are as follows:

Species	Number Allowed	Number Used	LSU IACUC #
Rats	240	33	1063

Investigator Signature

ABSTRACT

The dopamine (DA) neurons of the ventral tegmental area (VTA) and their mesoprefrontal projections have been implicated in the mediation of stress. Repeated stress can cause significant alterations of genomic response and behavioral "sensitization" of DA neurons of VTA and provide the substrate for post-traumatic stress syndrome. The experiments proposed will analyze the neurochemical alterations leading to "sensitization" of VTA DA neurons resulting from acute and repeated administration of the anxiogenic drugs FG 7142 and nicotine.

Injections of FG 7142 and nicotine induced the expression of *c-fos* gene in the Caudal Linear subnucleus (CLi) of VTA. The CLi contains DA neurons. CCK and NT are colocalized within these DA neurons. The NT mRNA is expressed more intensely in CLi than CCK mRNA. NT and CCK play a crucial role in regulating the rate of synthesis, turnover and release of dopamine by the DA neurons of CLi.

This study will provide a quantitative analysis of the sequential neurochemical changes in CLi and VTA after acute and repeated administration of FG 7142 and nicotine. Experiments to analyze the details of these DA regulating factors are critical towards our understanding of the mechanisms underlying sensitization of DA neurons to repeated stress.

STRESS, DOPAMINE AND OPIATE RECEPTORS

INTRODUCTION

The dopamine (DA) neurons of the ventral tegmental area (VTA) and their mesolimbic and mesostriatal projections have been strongly implicated in the mediation of stress. Several studies using established experimental methods for inducing stress showed a pronounced increase of DA utilization in the mesoprefrontal system, nucleus accumbens and the striatum (1,2,4,7,8,10,11,14,15,18,24,36), but these studies could not establish the specific source of the increased DA convincingly (7,8,10,11,14,15,18,24,36). Only recently Deutch et al., (10) showed that the c-fos gene is induced prominently in the CLi subdivision of VTA by restraint and injections of FG 7142, a potent anxiogenic drug, and the levels of dopamine increases significantly in the mesoprefrontal system after acute injections of FG 7142. Results from preliminary experiments performed in our lab confirms the above observations of Deutch et al.,(10) that after injections of FG 7142 as well as nicotine, yet another drug consumed in association with acute stress, Fos reactive cells are prominently noted in the caudal linear nucleus of VTA, thereby bringing the role played by CLi in transducing the effects of stressors into focus.

The common theme among many of these studies is that stressful stimuli stimulate the VTA DA neurons and increase synthesis and release of DA at their mesoprefrontal and mesolimbic target areas. However, varieties of other factors, different neurochemical inputs to these DA neurons, for example, can modify the excitable state of the DA neurons and alter the rate of synthesis and release of DA from these cells. Yet another key factor appears to be the role played by NT and CCK, peptides colocalized within the DA neurons, in

regulating the rate of synthesis, turnover and release of dopamine by the DA neurons of CLi.

The VTA subnucleus CLi, a part of the mesoprefrontal system, is connected to the anterior limbic and the entorhinal cortex, locus ceruleus, amygdala and to the lateral septal nucleus (35). CLi contains DA neurons (16), and DA neurons colocalized with cholecystokinin (CCK) and neurotensin (NT). The number of neurons as well as the intensity of expression of mRNA of NT is higher in CLi than any other VTA subnucleus (16). Acute injections of the anxiogenic FG 7142 and nicotine induce Fos in the CLi subdivision of VTA. Fos, the protein encoded by the c-fos gene (11), along with a protein product of yet another immediate early gene, Jun, binds with the AP-1 binding site of many other genes to result in increased levels of many neuropeptides. It is of interest to note that both CCK and NT genes have been demonstrated to contain the AP-1 binding site (12,20,21).

Collectively these results suggest that the metabolism, turnover rate, synthesis and release of dopamine from CLi may be modified by the intrinsic levels of the NT and CCK within the CLi neurons.

The major focus of these research project is to conduct experiments to analyze the details of how acute and repeated injections of anxiogenic drugs affect these DA regulating factors in the DA containing neurons of the VTA. This knowledge is critical towards our understanding of the response by the mesoprefrontal system to stressor agents.

NARRATIVE

The experiments conducted during the first year were directed to the understanding of the effects of single injections of FG 7142 and Nicotine on dopamine neurons of VTA.

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1. Experiments to identify stress responsive areas in VTA:

Introduction: Since the DA neurons of VTA are subdivided into five subnuclei, we conducted several additional experiments, using the pattern of induction of the gene c-fos, to a) identify whether a single subnucleus of VTA is involved or more than one subnucleus is involved in the mediation of stress effects and b) If more than one subnucleus is involved, identify which division(s) is (are) more involved in acute response to FG 7142, an anxiogenic agent. Induction of c-fos has been used as a marker (13), with single-cell specificity, to map & follow the temporal and spatial involvement of certain subsets of neurons following seizures, stress, amphetamine & stress responsive responsive striatal cells.

Materials and methods: Experiments were conducted in several groups of male Sprague-Dawley rats (250-350gms). 1. Rats which received the vehicle (saline 1.0ml) alone (n=2). 2. Experimental rats received injections of the anxiogenic drug FG 7142 alone (n=4) this is in addition to four rats that received 20mg/kg of FG 7142 for preliminary studies. .

The control and treated rats were anesthetized with pentobarbital, perfused intracardially with 4% paraformaldehyde in sodium phosphate pH 7.4. The brains were postfixed in 6% sucrose in phosphate buffer. The brains were cut into 40μ thick sections and were then processed for ABC

immunohistochemical technique with 1:500 concentration of an antiserum to Fos protein for 48hrs and DAB as the chromogen. The sections were studied with darkfield and brightfield microscopy and projection drawings were made using camera lucida. The various nuclei of the midbrain and especially the ventral tegmental areas were defined in adjacent sections counterstained with cresyl violet. The atlas of Paxinos and Watson was used to identify various mesencephalic nuclei.

Results: Injections of 20mg/kg of FG 7142 resulted in labeling few cells in the nucleus parabrachialis pigmentosus, but most prominently in the caudal linear nucleus. The rostral linear, interfascicular and paranigralis did not contain any Fos reactive cells. The Fos immunoreactive cells are also prominently noted in the supramammilllary nucleus. Fos reactive cells were absent in any other midbrain nuclei.

Conclusions: These additional cases confirm the observations of Deutch et al.,(10) that CLi subdivision of VTA may play a major role mediating stress response, and also our results from earlier preliminary experiments.

Experiments to evaluate the possibility that the DA neurons of the VTA may be activated by stressful stimuli either by stimulation of the NMDA receptors or by inhibition of the GABA receptors:

Introduction: One of the important hypotheses to be tested in the study is to evaluate the possibility that the stressor agents stimulate CLi DA neurons and lead to Fos induction and ultimately to increased dopamine synthesis and release either by activation of the NMDA receptors or by disinhibition of GABA receptors found on the DA neurons.

Materials and methods: Experiments were conducted in several groups of male Sprague-Dawley rats (250-350gms). 1. Rats that received the vehicle (saline 1.0ml) alone (n=2). 2. Experimental rats that received injections of 1.0mg/kg of MK 801 alone (n=3); 3. Rats that received injections of 4mg/kg of CPP alone (n=2); 4. Rats that received injections of 1.0mg/kg of MK 801 alone (n=3);the anxiogenic drug FG 7142 30 min after 1.0mg.kg of MK 801 and 5. Rats that received injections of 20mg/kg of i.p FG 7142 30 min after 4.0mg.kg of CPP alone (n=2).

Results: Injections of MK-801, a non specific open channel blocker of the NMDA receptors or CPP, a specific antagonist of the NMDA receptor alone resulted in labeling with Fos-Li in many neurons of several cortical areas and septal nucleus (LS) but none in CPu or nucleus accumbens (Acb) or VTA. After injections of 20mg/kg of i.p FG 7142 and 0.8mg/kg of s.c., nicotine following either MK 801(B) or CPP (C), Fos reactive neurons are seen in several cortical regions, but much less prominently than with either MK 801 or CPP alone, but these specific and nonspecific NMDA antagonists completely blocked nicotine induced Fos activation in the VTA.

Conclusions: It is concluded that FG 7142 and nicotine induced Fos activation in CLi is mediated by the NMDA receptors and that Fos activation may be abolished by NMDA receptor antagonists. These results confirm the hypothesis that stimulation of glutamate (NMDA) receptors located on the VTA DA neurons are critical for the activation of these stress responsive neurons by anxiogenic agents. Interestingly injections of MK 801 alone resulted in prominent labeling of Fos activity in the paraventricular nucleus of the hypothalamus, an area of the brain well recognized to respond to stress by increasing the synthesis and release of CRF and ACTH. It is of interest to note

that MK 801 injections have been shown to increase the release of CRF and ACTH. Further details of these experiments are still under evaluation.

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3. The pattern of Fos induction in the hypothalamus by FG 7142 and Nicotine:

Introduction: There have been very few studies to identify the specific subnuclei of VTA that respond to stressful stimuli. Deutsch et al, using a combined neuroanatomical and neurochemical approach showed, for the first time in a convincing manner, that the caudal linear subnucleus of the VTA may be involved in stress mediation. Since this is a brand new observation, it was felt by the P.I that there was a need to correlate the neurochemical changes in CLi with the neurochemical changes in at least one other area of the brain that is well established to mediate stress response. The paraventricular nucleus (PVN) of the hypothalamus, which responds to stressful stimuli by increased synthesis and release of corticotrophin releasing factor (CRF) and ultimately responsible for the release of ACTH, was included in the analysis of results in these acute experiments.

To extend our understanding of FG 7142 and nicotine and their interactions with various hypothalamic nuclei that might mediate stress response, we mapped the pattern of expression of the gene c-fos in the hypothalamus as an important marker of some of the earliest changes occurring at gene transcription level after acute nicotine injections.

Materials and methods: These experiments did not require the use of any additional rats. Sections from hypothalamic levels of brain from above mentioned experiments were used to analyze the pattern of distribution of Fos in stress responsive hypothalamic areas.

Results: "Control" group: In saline and drug-naive as well as saline-injected control rats, neurons with Fos-Li were rarely seen in the anterior and mid hypothalamic levels, but many cells with Fos-Li were noted in the septal nuclei, supramamillary region, and peripeduncular areas. (not shown in Figures). Nicotine group: At the anterior hypothalamic levels, Fos reactive neurons were prominent in the medial and lateral preoptic region, the lateral hypothalamic areas and also in the bed nucleus of stria terminalis (Figs.1A & 2A). Among the various subdivisions of the paraventricular nucleus of the hypothalamus, the medial parvocellular division showed the maximum number of Fos reactive neurons. Within the magnocellular PVN, many Fos reactive cells were noted in the periphery of the nucleus, but the central core of the magnocellular PVN was free of neurons with Fos-Li. In the supraoptic nucleus Fos-Li was noted mostly in the superficial regions of the nucleus, but only after injections of 1.0 mg/kg or 1.4mg/kg of nicotine (Figs. 1B,C & 2B,C,D). The arcuate nucleus and the lateral hypothalamic area also contained significant number of Fos immunoreactive neurons (Figs 1D & 2E).

At caudal levels Fos reactive cells were seen in the supramamillary regions also prominently in the nucleus reuniens of the thalamus (Figs. 1E & 2F). Of significance is that the suprachiasmatic nucleus, anterior and the ventromedial regions of the hypothalamus and the different subnuclei of the mammillary nucleus were relatively free of Fos reactive neurons. Nicotine induced c-fos expression was clearly blocked by 1mg/kg of mecamylamine, an antagonist for central nicotinic receptors, administered 30 min prior to nicotine injections. FG-7142 group: Interestingly FG 7142 did not induce Fos activity in the PVN. Further details of this study in under analysis.

Conclusions: These results suggest that acute injections of nicotine, a drug considered to be anxiolytic, acutually induce intense Fos expression in the PVN of the hypothalamus, a nucleus which is well recognized to respond to acute stress by CRF and ACTH release, two neurochemicals which mediate acute stress respo neeurohumoral responses to acute stress.

Publication: Preliminary results from some of these experiments were presented in the meeting of The International Symposium on Nicotine, held on July 21-24, 1994 at Montreal , Canada. The abstract is enclosed in the Appendix section.

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4. Experiments to establish the qualitative and quantitative aspects of expression of CCK, NT and TH mRNAs in VTA in control and in animals that received single injections of saline and FG 7142 and nicotine.

Introduction: Biochemical studies suggest that after acute stress the turnover of DA is increased as reflected by an increase in the turnover of TH, the rate limiting enzyme in the synthesis of DA (10,14). CCK and NT, two neuropeptides colocalized within the DA neurons of VTA, have significant role in the synthesis, turnover and release of DA in the nucleus accumbens (16,22-27,28-34). As an initial and necessary step towards understanding the effects of acute and repeated FG 7142 and nicotine injections on the TH, CCK and NT mRNA and their interactions with VTA nuclei, we have mapped and performed quantitative analysis of the pattern of expression of mRNAs of TH, CCK and NT in the ventral midbrain. The results will allow us to provide the baseline information that is necessary for the evlauation altered expression of TH, CCK and NT genes in CLi after repeated administation of FG 7142 and nicotine. Experiments using an oligonucleotide probe directed against the nucleotide

sequence of 1441-1485 of rat TH cDNA have been completed and the preliminary results are reported here.

Materials and methods: Male Sprague-Dawley rats (250gms) were housed four per cage in room illuminated from 8.00 A.M to 8.00 P.M. with free access to water and food. The initial set of experiments were performed to evaluate the effects of a single injection of 20mg/kg of the anxiogenic agent FG 7142 and 1.0mg/kg of nicotine.a) tissue preparation: The control and treated Sprague-Dawley rats (250gms) were anesthetized with pentobarbital and undergo intracardiac perfusion with PLPG solution four hours after the injctions. All sacrifice procedures were performed between 10.00 A.M and 12.00 Noon. Every five out of 10 frozen midbrain sections were thaw-mounted onto slides which are pretreated with Denhardt's solution, coated with poly-L-lysine and acetylated. These sections were used for in situ hybridization technique according to the method of Uhl (37-40).

b) Synthesis of oligonucleotide probes: A 40 to 50 base mRNA-sense template oligonucleotide corresponding to rat mRNA of interest and an antisense 12-base cDNA "primer" complementary to regions beginning approximately 5 bases from the 3' end of the "template" strand DNA were synthesized (37-40). These complementary sequences were allowed to hybridize to each other, and the antisense strand extended by using Klenow fragment of DNA polymerase I, 35S-labeled dCTP and dATP and unlabeled dGTP and dTTP. Radiolabeled product were separated from the template using a 12% polyacrylamide gel electrophoresis under denaturing conditions and eluted with boiling water. Specific activities of each probe varied from 10000 to 20000 ci/mmol.

Probes for NT, CCK, and TH mRNAs: The NT probe is directed against nucleotide sequence 443-487 of rat NT gene (17) and the CCK probe against the nucleotide sequence 451to 491 of rat CCK gene (17). The nucleotide sequence of 1441-1485 of rat TH cDNA Berod, Young and Hokfelt (3,41) was used to synsthesize TH probe.

c) Hybridization and washing: 25ul of 35S-labeled probe will be applied to each section in a hybridization buffer with 0.8M NaCl and 60% deionized formamide. Autoradiograms will be generated by dipping slides in Kodak NTB-2 emulsion. Following appropriate duration of exposure, the emulsion will be developed, the tissue stained with toludine blue, the slides mounted with Permount, and the sections will be analyzed using bright- and dark-field microscopy. Several "control" measures were done to satisfy many criteria for specificity.

d). Data collection, analysis and interpretation: The sections were analyzed for automated counting of autoradiographic grain counts/ cell that express the different mRNAs in VTA. The image analysis sytem is IBM PC based and is a product of Bioscan Inc. Seattle. The image acquiring system consists of a MOS camera which is attatched to an Overlay frame grabber. The captured image is displayed in a Sony color monitor. This image was analyzed with a software OPTIMAS which runs under Windows. The actual process of the grain counting is performed using a macro written specially for counting autoradiographic grains/cell. The raw data obtained from these experiments will be stored automatically into Microsoft Excel with a specially written subprogram. Using these programs the data were analyzed with both parametric and non-parametric methods. The Student's t-Test will be used to compare the number of cells expressing mRNA within a subnucleus of VTA (19,

37,39). The data will also be analyzed with Kolmogrov-Smirnov method of analysis.

Results: The analysis of results from these experiments are still in progress. Grain counting using "Optimas" system, a IBM 486 based computer assisted image analysis system, shows that the density grain/cell in the SNpc was about two and half times higher in the rostral and medial most area of SNpc than the most caudal regions. There was also a gradient mediolaterally with neurons located in the medial most areas of SNpc contain the highest grain density/cell whereas neurons in the lateral areas of SNpc contain moderate density of labeling with 35S TH oligonucleotide probe (Fig. 4 A & B).

The results are remarkably similar to the pattern of distribution as well as grain density/cell of the CCK mRNA in the substantia nigra pars compacta. The preliminary findings from grain counting in VTA suggests that the interfascicular nucleus contains the most dense accumulation of autoradiographic grains, the nucleus parabrachialis pigmentosus is the next and the paranigralis, rostral and caudal linear nuclei have less dense grains/cell.

CONCLUSIONS

1. The anxiogenic agent FG 7142 and nicotine induce the c-fos gene most prominently in the CLi subnucleus of VTA, thereby supporting the hypothesis that among the different subdivisions of VTA, the caudal linear subnucleus is specifically involved in transducing the effects of stressor agents.

2. CLi contains neurons that express TH mRNA only and cells that express TH, CCK, NT mRNAs. The expression of NT mRNA is more intense in CLI than CCK.

3. The quantitative and qualitative studies of the patterns of expression of TH, CCK, and NT mRNAs in control animals suggest, that the different subnuclei of the DA neurons of VTA and SNpc have varying degree of expression of these individual mRNAs of interest.

4. The induction of Fos in the different subnuclei of VTA by FG 7142 and nicotine is mediated through stimulation of the NMDA receptors in these neurons. FG 7142 may result in stimulation of NMDA receptors by an indirect action, namely by inhibiting GABAergic receptors.

5. From these early studies it is clear that increased dopamine turnover and increased locomotor activity which reflect the clinical syndrome of stress that is noted with acute injections of FG 7142 and nicotine may arise from different neurochemical interactions.

6. The results from experiments conducted during the first year have provided all the normative data that are required to proceed with next set of experiments, which consists of determining the neurochemical alterations in VTA noted 16 hrs, 72 hrs and 8 days after acute injections of these anxiogenic agents.

CHANGE IN DIRECTIONS:

At the present time I do not see any reasons to change the directions or the goals of the studies. But two very surprising and intriguing results remain to be further evaluated.

1. Nicotine is well recognized to be an anxiolytic agent. But much to our surprise, acute nicotine injections, stimulate the paraventricular nucleus of the hypothalamus (PVN) intensely in a dose dependent fashion, resulting in an intense expression of c-fos. This area of the hypothalamus is the most significant site of response to acute stress in the entire CNS.

2. It was also surprising to note that MK 801, a nonspecific antagonist of NMDA receptors, caused a profound expression of Fos in PVN, whereas MK 801 and CPP block expression of Fos in every other areas of the brain so far studied. This observation is consistent with the biochemical observations that MK 801 induces releases ACTH profoundly.

Even though these two results are unexpected, the results from the VTA studies confirm our expectations and hypotheses and experiments to analyze neurochemical changes after repeated acute and repeated injections of FG 7142 and nicotine will continue as planned.

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APPENDIX

International Symposium on Nicotine: The Effects of Nicotine on Biological Systems II

Satellite Symposium of the XIIth International Congress of Pharmacology, Montreal, Canada, July 21–24, 1994

The Abstracts

Edited by
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NICOTINIC RECEPTOR SUBTYPES CONTROLLING THE SECRETAGOGUE AND MITOGENIC EFFECTS OF NICOTINIC AGONISTS IN SMALL-CELL LUNG CARCINOMA CELL LINES

P 51

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We have recently described the presence of neuronal type nicotinic receptors of an α-Bungarotoxin (αBgtx) binding sites in human small cell lung carcinoma (SCLC) cell lines (B. Chini et al., 1992; P. Tarroni et al., 1992). These receptors mediate both the secretagogue and mitogenic effects of nicotine in these cancer cells (M.G. Cattaneo et al., 1993). We now found that different related polypeptide nicotinic antagonists (αBgtx, α-Conotoxin MI (αCtx) and κBungarotoxin (κBgtx)) are all potent blockers of both the secretagogue and mitogenic effects of nicotinic agonists in SCLC cells. Nicotine and Cytisine stimulate [^{3}H]Serotonin ([^{3}H]5HT) release from three different SCLC cell lines (NCI-H-69, NCI-N592 and GLC8) in a time- and dose-dependent manner, the EC_{50s} being 20±2, 22.5±1 and 23.5±1(nicotine) and for the two drugs, respectively. Nicotine-induced [^{3}H]5HT release was completely dependent on the presence of external Ca^{2+}, suggesting that Ca^{2+} influx is a crucial step in this phenomenom. αBgtx, αCtx and κBgtx antagonized nicotine- and cytisine-induced [^{3}H]5HT release in a dose-dependent manner (IC_{50s} of 1 nM, 10 pM and 1 pM, respectively).
Nicotine- and cytisine-stimulated SCLC cell proliferation was also completely prevented by αBgtx 1.53 μM and αCtx 1.1 μM, while the mitogenic effects of serotonin (M.G.Cattaneo et al., 1993) were not affected. Besides the α_3, α_5 and β_2 nicotinic subunits we have already described in SCLC cells, we have now found evidence, by PCR analysis, for the presence in SCLC cells of the α_7 and β_4 nicotinic subunits. β_4 is known to confer cytisine sensitivity to neuronal-type nicotinic receptors, and α_7 is known to code for the most abudant αBgtx-sensitive nicotinic receptor in the nervous system.
Our results suggest that the αBgtx-sensitive nicotinic receptors of SCLC cells, possibly coded by the α_7 subunit, play a crucial role in mediating the biological effects of nicotine in this very aggressive lung cancer.

Section 7: Effects on gene expression

NICOTINE INDUCES FOS INTENSELY IN THE PARVOCELLULAR PARAVENTRICULAR NUCLEUS, THE PREOPTIC AND THE LATERAL HYPOTHALAMUS IN RATS.

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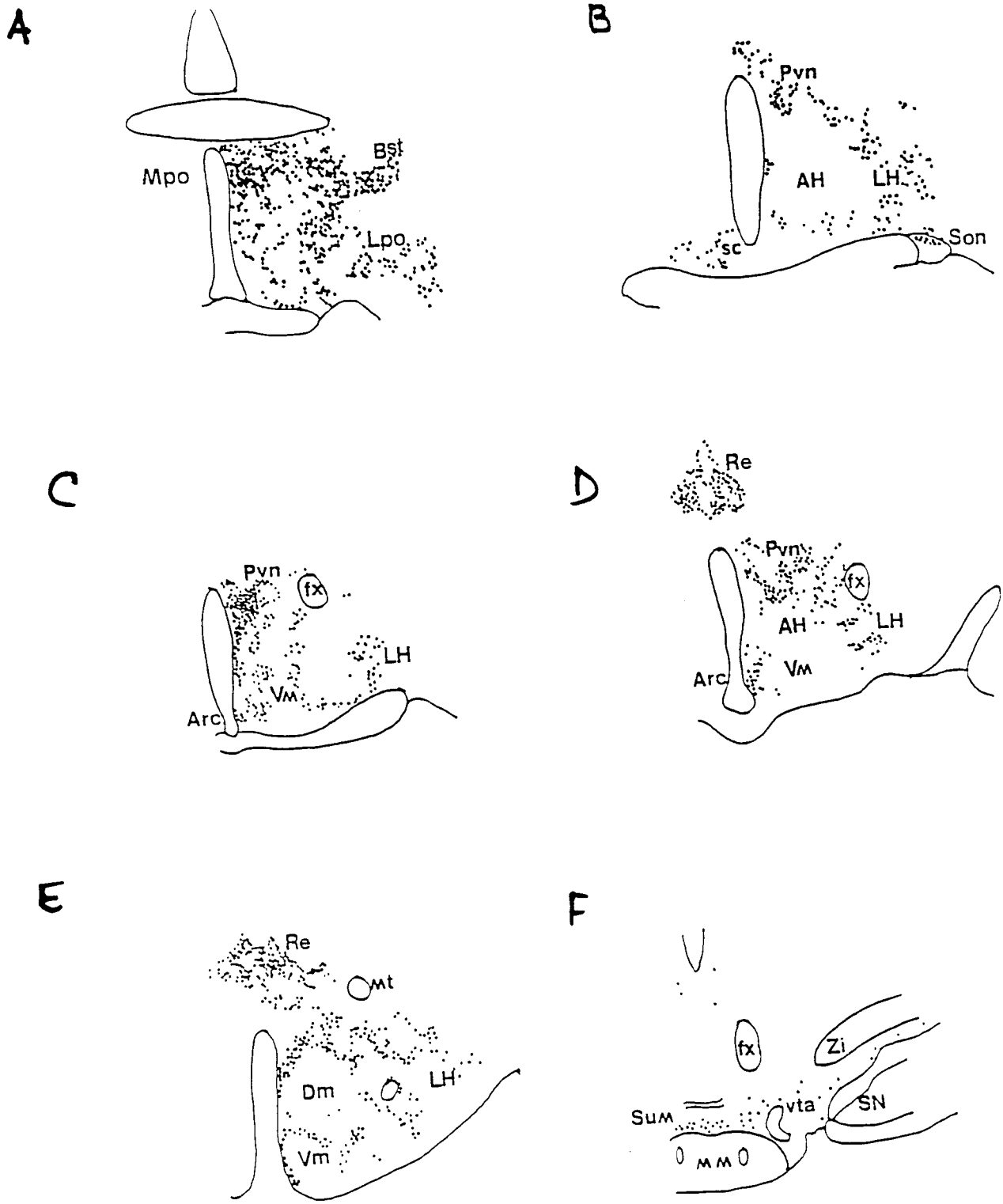
Subcutaneous injections of nicotine (0.4 to 1.0 mg/kg) in adult male rats resulted in the induction of c-fos gene most prominently and selectively in the parvocellular, but not the magnocellular subdivision of the hypothalamic paraventricular nucleus. A significant number of neurons in the superficial regions of the supraoptic nucleus also contained Fos reactivity, but only with larger doses (1.0 mg/kg) of nicotine. Fos immunoreactive neurons were also prominent in the supramammillary regions. The lateral preoptic area, the anterior and posterior aspects of the lateral hypothalamus contained significant number of Fos immunoreactive neurons. The medial preoptic area, the suprachiasmatic and the periventricular nuclei of the hypothalamus were relatively free of Fos reactive neurons. Injections of mecamylamine completely abolished nicotine induced Fos immunoreactivity in all of these cases. These results suggest that acute injections of nicotine induce intense Fos expression in two major areas of the rat hypothalamus, namely 1. the CRF neurons of the parvocellular paraventricular nucleus, an area of the hypothalamus recognized to mediate stress response and 2. the lateral preoptic areas and the lateral hypothalamus, regions of the hypothalamus strongly implicated in intracranial self-stimulation behavior. Supported by The Smokeless Tobacco Research Council, N.Y., and the Department of Defense, U.S.A.



PATTERN OF INDUCTION OF FOS IN THE HYPOTHALAMUS OF RATS.
The animals received 0.8mg/kg of Nicotine s.c.,

Fos reactive neurons in the paraventricular nucleus (C) and the supraoptic (4)
nucleus after injections of 0.8mg.kg of s.c. nicotine. (Details in text)